

A NOVEL β_1 -BLOCKER BASED ON ANTISENSE OLIGONUCLEOTIDES:
A NEW GENE THERAPY APPROACH IN HYPERTENSION,
CARDIAC HYPERTROPHY AND MYOCARDIAL ISCHEMIA

By

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Dedicated to my husband Ryan, and my parents.

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Abstract of Dissertation Presented to the Graduate School
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Objectives: β -Adrenoceptor antagonists (β -blockers) are effective in treating hypertension, heart failure and myocardial infarction, but they have poor patient compliance because of the daily regimen and side effects such as central nervous system (CNS) reactions and nonspecific inhibition of β 2-adrenoceptor (β 2-AR). To overcome these problems, I have developed a novel and specific β 1-blocker by targeting β 1-adrenoceptor (β 1-AR) mRNA with antisense oligonucleotides (β 1-AS-ODN) and optimized systemic delivery with cationic liposomes. The effects of β 1-AS-ODN on blood pressure, left ventricular hypertrophy (LVH) and myocardial ischemia were tested in spontaneously hypertensive rats (SHR) and isolated rat hearts subjected to ischemia/reperfusion. The antihypertensive mechanisms were investigated and the safety profile of repeated administration of β 1-AS-ODN was studied.

Results: A single intravenous injection of 0.5-1 mg/kg β 1-AS-ODN with cationic liposomes significantly decreased blood pressure by 25-35 mmHg for 20-33 days in SHR ($P<0.05$), with no effect on heart rate. β 1-AS-ODN significantly reduced the β 1-AR levels in the heart and kidney, without affecting β 2-AR. The mechanisms for blood pressure reduction appeared to involve a rapid decrease in cardiac contractility and a delayed but marked reduction in plasma renin activity. Quantitative autoradiography revealed no changes in β -AR in the brain, indicating the absence of CNS effects. β 1-AS-ODN is effective with repeated injections. It not only produced a sustained reduction in blood pressure, but also significantly reduced LVH in SHR. No toxicity (assessed by liver transaminases, hematology, and tissue histopathology) or immune response was observed during long-term treatment. In addition, pretreatment with β 1-AS-ODN significantly attenuated cardiac dysfunction and lipid peroxidation of isolated rat hearts induced by ischemia-reperfusion.

Conclusion: Systemic injection of β 1-AS-ODN delivered by cationic liposomes can reduce high blood pressure and cardiac hypertrophy, and can protect the heart from ischemic injury. It is clearly longer-lasting than currently available β -blockers and likely to have fewer side effects because of its β 1-specificity and lack of CNS effects and bradycardia. Repeated administration is effective without causing toxicity or immune stimulation. Therefore β 1-AS-ODN is viable as a potentially new prolonged antihypertensive agent with benefits for cardiac remodeling and myocardial protection from ischemia.

CHAPTER 1 INTRODUCTION

1.1 β_1 -Adrenoceptors

The sympathetic nervous system (SNS) plays an important role in the regulation of a wide variety of physiological functions that are essential for survival and quality of life. Most postganglionic sympathetic nerve terminals release norepinephrine as a neurotransmitter, which exerts its effects through interaction with adrenoceptors. Adrenoceptors (AR) were first subdivided into α and β subtypes by Ahlquist in 1948 (1). β -AR was further classified into β_1 and β_2 subtypes in 1967 (2) based on their relative potencies for the endogenous agonists adrenaline (epinephrine) and noradrenaline (norepinephrine). A third subtype β_3 was discovered in 1989 (3). β_1 -AR is the predominant AR subtype expressed in the myocardium and renal cortex. When activated, β_1 -AR induces positive inotropic, chronotropic and lusitropic responses to catecholamines in the heart, i.e. an increase in myocardial contractility, heart rate and relaxation speed. It also stimulates renin release from the juxtaglomerular apparatus in the kidney and upregulates the peripheral renin-angiotensin system (4,5). Therefore the combined effect of β_1 -adrenergic activation is an elevation in cardiac output and blood pressure. β_1 -AR is essential for normal cardiac performance. Its

importance is manifested by the prenatal death of β_1 -AR knockout mice (6) and by heart failure associated with impaired β_1 -AR functions (7,8).

β_1 -AR belongs to the superfamily of G-protein-coupled receptors that have seven hydrophobic transmembrane domains. The ligand-binding site is located in the transmembrane regions (9), and the third intracellular hydrophilic loop appears to be most important for coupling β_1 -AR to G protein (10). The coding sequence of human β_1 -AR is 1434 bp long and contains no introns (11). Although β_1 -AR has been characterized for many years, the understanding of its intracellular signal transduction has largely derived from the study of β_2 -AR. The putative signaling pathway for cardiac β_1 -AR involves stimulatory G protein (Gs), activation of adenylyl cyclase (AC), accumulation of cAMP, stimulation of cAMP-dependent protein kinase A (PKA), and phosphorylation of key target proteins (including L-type calcium channel [$I_{Ca,L}$], phospholamban and troponin).

β_1 -AR undergoes desensitization and down-regulation when exposed to the agonists. Rapid desensitization occurs after acute activation (within minutes) which appears to be through receptor phosphorylation and sequestration (12). Chronic activation leads to receptor down-regulation which involves reduced mRNA levels and enhanced degradation of β_1 -AR (13). Diminished density and functional responsiveness of cardiac β_1 -AR are common features of chronic heart failure and myocardial infarction that result from an excessive adrenergic drive in these diseases (7,14).

1.2 Hypertension: Definition, Prevalence and Risks

Hypertension, manifested by chronic high blood pressure, affects a large population and is a silent killer leading to severe cardiovascular problems. The conceptual definition of hypertension, according to Kaplan in his *Clinical Hypertension* (15), is "the level of blood pressure at which the benefits (minus the risks and costs) of treatment exceed the risks and costs (minus benefits) of inaction." Numerically, hypertension is defined by the World Health Organization (WHO) as the blood pressure (BP) higher than 160/95 mmHg (systolic/diastolic BP). However, hypertension is treated more aggressively in the United States with the clinical end point <140/90 mmHg. It is estimated that hypertension afflicts one fourth of adults in America, i.e. 50 million people (16,17), and is the No. 1 reason for office visits to specialists in internal medicine (18). However, due to the asymptomatic nature of this disease, many patients are largely unaware of high BP and therefore have little motivation to seek or follow treatment. As a result, only about 50% of hypertensives in the U.S. get their BP under control.

The danger of hypertension does not lie in high BP itself, but in the complications of high BP. Hypertensives generally suffer premature disability or death from cardiovascular diseases, because chronic untreated high BP increases the incidence of deadly events such as stroke, heart failure, myocardial infarction and renal dysfunction (16,19). The mechanisms are complex and may involve the structural changes in blood vessels and cardiac

tissues termed hypertrophy and remodeling, which eventually lead to organ damage and functional insufficiency (20).

1.2.1 Hypertension: a Major Risk Factor for Heart Failure

Hypertension and heart failure are closely associated. Heart failure, a pathophysiological state whereby the heart fails to pump sufficient blood to meet the oxygen requirements of the body, is life-threatening and affects 4 million Americans (21). The prevalence is rising with 400,000 new cases every year, mostly in patients over 65 years old. The best available data on hypertension as a prelude to heart failure were derived from the Framingham study (22). This study involved follow-up of more than 5,000 patients for a median of 14 years, and found that hypertension is the largest single risk factor for the development of heart failure, accounting for 39% of attributable risk in men and 59% in women. How hypertension degenerates into heart failure is not completely understood. However, it is well accepted that the heart responds to a prolonged pressure overload in three major ways (23): 1) left ventricular hypertrophy (LVH), 2) adrenergic stimulation of heart rate and contractility, and 3) volume expansion. Mild hypertension increases the risk of LVH by 2-3 times, and severe hypertension leads to tenfold elevated risk (24). LVH is initially a compensatory response to hypertension which helps maintain normal cardiac performance, but gradually progresses to cardiac remodeling and dysfunction. An overactive adrenergic system, indicated by an increase in norepinephrine (NE) release (25) and sympathetic nerve traffic (26,27), has been observed in the early stage of

essential hypertension and results in the diminished responsiveness of cardiac β -adrenergic receptors in failing hearts (7).

1.2.2 Hypertension: a Major Risk Factor for Myocardial Infarction

Hypertension is also a major risk factor for myocardial infarction. Myocardial infarction occurs when there is an abrupt decrease in the coronary blood flow after the thrombotic occlusion of a coronary artery previously narrowed by atherosclerosis. Approximately 1.5 million myocardial infarctions occur in America every year, with about 30% mortality rate. In the Framingham Heart Study, 30-year follow-up data revealed that the incidence of myocardial infarction was proportional to the level of systolic BP in a wide range from normotension to severe hypertension (28-30). Active treatment of isolated systolic hypertension in the elderly resulted in a 27% reduction in myocardial infarction and coronary heart disease (CHD)-related mortality (31). The mechanisms by which high BP increases the risk of CHD are yet unclear. Extensive studies on hypertensive rats revealed that Ca^{2+} is elevated excessively in myocytes and coronary artery wall of these animals. The Ca^{2+} overload increases the incidence of coronary spasm, which occurs in the early phase of acute myocardial infarction (32). Systemic hypertension also induces LVH, which reduces coronary flow reserve and renders the heart vulnerable to ischemia-induced damage (33). In addition, chronic sympathetic activation leads to endothelial dysfunction (34) and vascular smooth muscle hypertrophy (20) that further promote the development of atherosclerosis.

1.3 β_1 -Adrenoceptors and Cardiovascular Diseases

1.3.1 Overactive SNS in Cardiovascular Diseases

Overactive sympathetic activity plays a crucial role in the pathogenesis of cardiovascular diseases, including essential hypertension, congestive heart failure and myocardial infarction (35-37). Nearly 30 years ago, Julius *et al.* (38) observed that a noticeable fraction of subjects with borderline hypertension had an increased sympathetic and a decreased parasympathetic drive. This finding has since been supported by a large body of evidence. A meta-analysis of all published data (25) showed that plasma NE levels as an indirect marker of sympathetic tone were significantly elevated in younger patients with essential hypertension than in normotensive individuals, which was accompanied by an increase in heart rate and cardiac output (39). Concomitantly, NE spillover rate (40-42) and muscle sympathetic nerve traffic (27,43) rise progressively with the development of essential hypertension. Likewise, elevated plasma NE levels were observed universally in symptomatic and asymptomatic heart failure (44,45). Myocardial infarction is also associated with reduced baroreflex and increased sympathetic activation (46). These data indicate the sympathetic overactivation as a key participant in the initiation and maintenance of these cardiovascular problems. β_1 -AR, a central mediator of SNS activity, is therefore unequivocally involved in these deleterious events.

1.3.2 β_1 -Adrenoceptors and Hypertension

β_1 -adrenergic activation induces positive inotropic and chronotropic responses in the heart and stimulates renin release in the kidney, thereby increasing cardiac output and upregulating the renin-angiotensin system (47). As a result, β_1 -agonists such as dobutamine can rescue acute cardiac failure by increasing myocardial contractility and heart rate (48). Pharmacological interventions by β -blockers are effective in reducing mortality in high risk arrhythmic patients and in lowering BP (49,50). All β -blockers can inhibit β_1 -AR and share structural similarity to β -agonist isoproterenol. Since the introduction of propranolol in 1964 (51), β -blockers have become the first line therapy for treating hypertension as recommended by the Joint National Committee and WHO. They are now proposed as the initial monotherapy for younger and middle-aged hypertensives, especially white males, and for patients with high levels of stress (52). The precise mechanisms by which these agents reduce BP remain unclear (53). However it is generally agreed that the primary effect of β -blockers is a reduction in cardiac output by 15-20%, resulting from the blockade of cardiac β_1 -AR, thereby reducing both heart rate and myocardial contractility (54). Besides, the inhibition of β_1 -AR in renal cortex suppresses renin production and release, which may lead to a diminished peripheral renin-angiotensin system (55). These concepts are supported by the observation that β -blockers are more effective for patients with high cardiac output and heart rate (52) and high renin profiles (56,57).

1.3.3 β_1 -Adrenoceptors and Heart Failure

The importance of fully functional β_1 -AR in the maintenance of normal heart performance is apparent, as the diminished functional responsiveness of β_1 -AR in chronic heart failure is closely correlated with the severity of impaired cardiac functions (7,58). Therefore β_1 -blockade was considered to contraindicate heart failure for a long period of time. However, early medical attempts to treat heart failure with β -adrenergic agonists showed only short-term benefits, and then precipitated symptoms with chronic use (59). This observation challenged the prevailing wisdom that β -adrenergic activation improved cardiac functions and therefore led to the pioneering trial by Dr. Hjalmarson's group in Sweden treating heart failure with β -blockers in 1975 (60). Since then, their success has been confirmed by overwhelming clinical and experimental evidence. These data suggest that in contrast to acute supportive effects, chronic β -adrenergic activation is detrimental to the heart (37,61).

Several recently completed multi-national, multi-center, large-scale clinical trials achieved clinical end points in heart failure patients on β -blockers, including improvement of systolic functions, exercise capacity and quality of life; and reduction in morbidity, hospitalization and mortality (62-67). In the Metoprolol in dilated Cardiomyopathy (MDC) trial (n=383), metoprolol treatment in patients with IDC resulted in a 34% reduction of the primary combined endpoint, total number of deaths and need for cardiac transplantation (62). In the US Carvedilol studies (n=1094), carvedilol reduced mortality by 65% in patients with ischemic

and idiopathic cardiomyopathy, which was highly significant (67). This led the Food and Drug Administration (FDA) to approve carvedilol for chronic heart failure. β -blockers are also effective in the prevention of heart failure in hypertensive patients. In the STOP trial (n=1627), old hypertensive patients treated with β -blockers (atenolol, metoprolol or pindolol) for an average of 25 months showed a significant reduction in heart failure rate and total death (68,69).

Many mechanisms have been proposed for the protective role of β -blockers in heart failure [reviewed in (70,71)]. They include the following:

- Improvement in cardiac function by restoring the density and functional sensitivity of β -AR that are desensitized and down-regulated after chronic sympathetic activation (62).
- Reduction in oxygen consumption and metabolic demand by decreasing heart rate (72,73), cardiac contractility and thereby ventricular workload.
- Regression of left ventricular hypertrophy (74,75).
- Inhibition of the renin-angiotensin system (23).
- Attenuation of cytotoxic and apoptotic effects of catecholamine (76,77).

Furthermore, recent advances in physiological genomics corroborate these clinical observations. Cardiac overexpression of human β_1 -AR (78), $G_{\alpha s}$ (79) or $G_{\alpha q}$ proteins (80) in transgenic mice improved contractile force only at a young age, then developed ventricular hypertrophy and systolic dysfunction, which ultimately resulted in a cardiomyopathic phenotype and premature death. The mechanisms are not clear, but growing evidence suggests that β_1 -AR

mediates the proliferative, cytotoxic (76) and apoptotic (77) effects of excessive NE on cardiomyocytes.

1.3.4 β_1 -Adrenoceptors and Myocardial Infarction

During acute myocardial infarction, excessive reflex activation of SNS kicks in and increases platelet aggregation, myocardial ischemia and arrhythmia (81). Elevated SNS activity has been considered an independent predictor for sudden cardiac death after myocardial infarction (46). β -blockers, by inhibiting cardiac β_1 -AR, can attenuate the detrimental effects of sympathetic activation and thus protect the heart from severe damage and ventricular fibrillation. Three clinical trials published in 1981 (82-84) demonstrated for the first time that β -blockers started after the onset of myocardial infarction reduced total mortality. Since then, over 55 randomized trials have been carried out in more than 55,000 patients. A summary of 24 long-term trials including about 25,000 patients showed that β -blockers reduced total mortality by 20% and sudden cardiac death by 34% over 2 years of follow-up after myocardial infarction (85). They also significantly decreased the incidence of recurrent infarction (83). It is now suggested that patients (especially older patients) with acute myocardial infarction, who have no contraindications to β -blockers, should be treated with early intravenous β -blockade followed by oral β -blockers indefinitely (81).

Many types of β -blockers including timolol, metoprolol, propranolol, acebutolol, pindolol, carvedilol and bisoprolol have been found to significantly reduce total mortality after myocardial infarction to similar extent. The property

they have in common is β_1 -blockade. Therefore β_1 -blockade appears to be the essential life-saving ingredient.

In animal models of ischemia/reperfusion, the mRNA level and density of β_1 -AR increased during acute infarction and decreased after chronic infarction (14,86-88). Pretreatment with β -blockers significantly reduced infarct size and prevented ventricular dysfunction compared with control animals (89).

The theoretical benefits of β -blockade in myocardial infarction include a decrease in oxygen demand, an antiarrhythmic effect, and a positive influence on myocardial metabolism (90). The decrease in oxygen demand results primarily from a reduction in heart rate and contractility. Excessive release of catecholamine during myocardial infarction can be arrhythmogenic. Sudden cardiac death accounts for almost half of mortality after myocardial infarction, which is due to myocardial tachyarrhythmia and fibrillation (91). β -blockers can prevent arrhythmia and are the only drugs that have consistently reduced sudden death in large trials (85). In addition, β -blockers prevent the derangement of cardiac energy metabolism. The cellular mechanisms underlying the beneficial effects of β -blockade in myocardial infarction are being studied extensively, which seem to involve the protection of intracellular Ca^{2+} homeostasis and protein phosphorylation (92), antioxidant changes (93), reduction in inflammatory cytokines (94), and favorable energy metabolism (95,96).

1.4 Why Antisense Gene therapy for β_1 -blockade?

1.4.1 Drawbacks of Current β -blockers

It is evident that β -blockers are reliable, successful and relatively safe in the control of hypertension, heart failure, myocardial infarction and arrhythmia. In spite of the success, this traditional drug therapy has many shortcomings. For example, the compliance for β -blockers in hypertensive patients has been less than 50% by some estimates (97). The reasons are multiple. First, the obvious asymptomatic nature of the initial phases of hypertension renders this disorder underdiagnosed and undertreated. Second, β -blockers are associated with a number of side effects due to nonspecific β_2 -antagonism (98) and central nervous system (CNS) reaction (99). Another problem with β -blockers, which is also true for all the available antihypertensive drugs, is short action, requiring a mandatory daily regimen. This further discourages patient compliance and produces a potentially dangerous peak-and-trough fluctuation of 24-hr BP (97).

β_2 -blockade

Most of the unwanted effects of β -blockers are caused by the blockade of β_2 -mediated functions, including bronchodilation, vasodilation, and mobilization of glucose and free fatty acids (98). Limited by pharmacological drug design, truly β_1 -specific antagonists have not been discovered, making these adverse effects inevitable even with highly β_1 -selective blockers at therapeutic doses (100).

Bronchodilation and vasodilation: Pulmonary bed and blood vessels express high levels of β_2 -AR, activation of which leads to bronchodilation and vasodilation. The suppression of this receptor leaves the α_1 -mediated vasoconstriction unopposed and results in elevated airway and vascular resistance (101,102). Therefore nonselective β -blockers are generally considered to contraindicate asthma and peripheral vascular diseases.

Blood sugar: β_2 -adrenergic activation promotes glycogenolysis and gluconeogenesis in the liver and thereby helps maintain normal glucose levels in the plasma. Nonselective β -blockers tend to decrease glucose tolerance. β_1 -selective agents are preferred over nonselective agents for patients with insulin-dependent diabetes mellitus, because nonselective β -blockers can increase the duration and severity of insulin-induced hypoglycemia, and β_1 -selective agents have a less pronounced effect on blood glucose levels (103,104).

Quality of life: Nonselective β -blockers such as propranolol markedly decrease quality of life when compared with the ACE inhibitor captopril (105). In contrast, β_1 -selective drugs cannot be differentiated from ACE inhibitors in terms of overall quality of life scores (105) and are superior to the calcium channel blocker nifedipine (106). In patients with chronic heart failure, β_1 -selective blockers showed significant advantages over nonselective agents in improving quality of life and NYHA class (62,63,65,66,107). The reason is that β_2 -mediated metabolic functions are largely unimpaired with β_1 -selective blockade (108,109). These functions, including mobilization of glucose and free fatty acids, are important in providing energy supply to and removing unwanted by-products from

exercising muscles. Therefore, post-exercise hypoglycemia is unlikely to occur, which contributes to lesser fatigue with β_1 -selective agents (110).

Blood lipid profile: Serum levels of total cholesterol and low-density lipoprotein (LDL) are generally not affected by β -blockers. But nonselective agents such as propranolol significantly increase triglyceride (TG) concentration and decrease high-density lipoprotein (HDL) levels (104,111), which have been associated with a higher risk of atherosclerosis (112-114). These effects are due to β_2 -blockade, as the changes are less apparent with β_1 -selective agents such as metoprolol and atenolol (110). With highly β_1 -selective agents such as bisoprolol, the changes appear to be minimal or absent (115).

CNS effects

In addition to the side effects induced by β_2 -inhibition, common complaints about β -blockers include depression, impotence, vivid dreams and insomnia (99). Because these effects are more pronounced with lipophilic agents that are able to cross the blood-brain barrier and enter the brain (116,117), they are generally attributed to the suppression of central β -AR functions (116).

Short action

Most β -blockers have short half-lives in plasma between 3 and 10 hr (90). Therefore a daily regimen is necessary. This problem is not unique to β -blockers, and all the antihypertensive drugs available today have to be taken at least once a day (97). Compulsory daily medication, along with adverse effects, discourages patients from staying on antihypertensive therapy.

These issues led us to conclude that the traditional pharmacological approach for β -blockade has reached a plateau. A new and innovative way must be considered to overcome these problems and to achieve better control of BP and heart dysfunction. Because hypertension and its cardiovascular complications are chronic disorders that affect a large population, any improvement in patient compliance may lead to significant reductions in morbidity and mortality in the long run and carry with it potential economical benefits to society.

1.4.2 Antisense Gene Therapy

Ideally, such a new therapy should provide β_1 -specific inhibition, and stable and persistent lowering of BP without CNS side effects or toxicity. Antisense gene therapy for β_1 -blockade holds promise for achieving all these goals.

The idea of using antisense oligonucleotides (AS ODN) to inhibit gene expression was first proposed and performed by Zamecnik and Stephenson in 1978 (118). Antisense oligonucleotides are short (typically 15-25 bases in length) synthesized and chemically modified DNA molecules that are complementary to the mRNA sequences encoding proteins of interest. They bind to mRNA transcripts in a specific manner by Watson-Crick base pairing and inhibit the expression of target proteins.

Several mechanisms of action have been proposed for AS ODN and demonstrated to work in different systems [reviewed by Crooke (119)]. One theory is translational arrest by assuming a steric hindrance to prevent ribosomal

assembly and read-through (120-122). A second mechanism works through activation of RNase H that degrades the RNA strand of an RNA-DNA duplex (123-125). Thus the mRNA is cleaved irreversibly and cannot be translated into proteins. In addition, AS ODNs have been designed to inhibit preRNA slicing (126) and to form triple helix with DNA (127), but these appear to be possible only in special cases.

In principle, an oligo of 12-15 bases long can provide sufficient affinity and specificity to its complementary mRNA (128). The specificity of AS ODN is so great that even two genes that differ by a mutation at a single base can be inhibited differentially (129). Therefore gene targeting specificity is the most obvious virtue of antisense strategy, as it is based on individual gene sequences. This also endows antisense with another advantage, which is versatility. Antisense can be designed to specifically intervene with the expression of any gene whose sequence is known. The design of AS ODN is rational and the production is inexpensive. Besides, AS ODNs are hydrophilic molecules that cannot cross the blood-brain barrier to cause CNS effects (130).

Specificity, simplicity and easy availability of synthesized oligos, along with developments in the human genome project, have made antisense technology a powerful tool for basic and clinical applications. It has been used increasingly to study gene functions in recent years (131-135). For example, transgenic mice expressing an antisense RNA against angiotensinogen (AGT) mRNA in the brain provided convincing data on the role of central AGT in the regulation of BP and fluid homeostasis (136). AS ODNs have also been explored as therapeutic

agents to suppress harmful components involved in disease development (137), including cancer (138,139), AIDS (140), autoimmune disorders (141,142) and restenosis (143-145). Its success is manifested by the recent approval of the first antisense drug, Vitravene, by FDA to treat cytomegalovirus-induced retinitis. Furthermore, antisense can be delivered to produce long-term gene inhibition and therapeutic benefits for treating cardiovascular diseases. Studies in our laboratories have established that systemic delivery of antisense cDNA directed at AT₁-receptor or angiotensin-converting enzyme by adeno-associated virus (AAV) results in a long-term attenuation (6 months) of hypertension in spontaneously hypertensive rats (146) as well as in double transgenic mice expressing human angiotensinogen and renin (147). The hypertension-associated ventricular hypertrophy and endothelial dysfunction are concomitantly reduced. Raizada's group (148,149) used retrovirus to deliver AT₁-receptor antisense which prevented the development of hypertension in SHR, and the normotensive phenotype was passed to the second generation. In addition, clinical trials are underway to evaluate the efficacy of AS ODN for treating cardiovascular problems such as restenosis (narrowing of blood vessels) after coronary angioplasty. These results indicate that antisense gene therapy is conceptually sound and technically feasible for the treatment of cardiovascular diseases including hypertension. Therefore a specific β_1 -adrenergic inhibition without CNS effects should be achieved by antisense molecules targeted to β_1 -AR.

1.5 Gene Delivery Vehicles (Viral vs. Nonviral Vectors)

It is evident that once a therapeutic target is defined, the success of gene therapy largely depends on the choice of the delivery system. An ideal vector should possess the following properties: (i) high efficiency of gene transfer; (ii) low toxicity, non-immunogenicity and non-mutagenesis; (iii) simplicity to produce on a large scale; (iv) maintained efficacy on repeated administration. Currently, numerous systems have been developed for gene therapy, which fall into two categories: viral and nonviral vectors.

1.5.1 Viral Vectors

The four viruses that have been broadly used for gene therapy are retrovirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus (HSV). The characteristics of these viral vectors are summarized in Table 1-1.

Although viral vectors are highly efficient in gene delivery, they suffer several significant drawbacks that make them currently inappropriate for clinical use, such as high acute toxicity, immunogenicity, risk for mutagenesis and insufficient pharmaceutical quantities (viral titers) (150-152). The safety issue has remained a major concern for viral vectors (153), and the critique became more intense after the tragic death of a young patient after receiving adenovirus-delivered gene therapy in 1999 (154,155). This incident alerts scientists of the immature status of current viral gene therapy and warrants more scrupulous and extensive study and improvement for viral vectors.

Table 1-1. Characteristics of presently available viral vectors for gene therapy.

	Retrovirus	AAV	Adenovirus	HSV
Insert size	Small (6.5kb)	Small (5kb)	Intermediate (8kb)	Large (36kb)
Viral titer	Low	Low	High	Low
Viral production	Helper-free	Helper	Helper-free	Helper/helper-free
Infectivity	Dividing cells only (except Lentivirus)	Broad	Broad	Broad
Integration	Chromosomal (random)	Chromosomal (not random)	Episomal	Episomal
In vivo risks	Mutagenesis	?	Immune response, cytotoxicity	Cytotoxicity
Latency	No	No	No	Yes

1.5.2 Nonviral Vectors

The latest development in nonviral vectors includes gene gun (156,157), cationic liposomes (158,159), polymer (160) and liposome/polymer complex (161). Some drawbacks are associated with these vectors, i.e. lower efficiency than viral vectors in gene transfer and transient gene expression. In spite of these limitations, nonviral vectors are now being pursued vigorously as a promising alternative for viral vector gene therapy, because of their low toxicity, non-immunogenicity and feasibility to be produced in large quantities [reviewed in (162,163)]. Liposomes, a traditional drug carrier used in pharmaceuticals, have established safety and pharmacological properties (164,165). They can also reduce the toxicity of many compounds such as doxorubicin and anthracycline

(166,167). In addition, cationic liposomes are effective vehicles for gene delivery (168) by facilitating cellular uptake of DNA, protecting DNA from degradation and extending the circulation time (169). For example, cationic liposomes are used routinely for *in vitro* transfection. Their therapeutic potentials as gene therapy vectors have been demonstrated in clinical trials of a variety of diseases such as cystic fibrosis (170) and cancer (171-173). Currently 75 clinical trials are using liposomal vectors for gene delivery which involve approximately 21% of all patients treated by gene therapy (174).

1.6 Specific Aims

β -blockers are used successfully to treat hypertension, heart failure and myocardial infarction. However they have poor patient compliance due to the mandatory daily regimen and side effects resulting from nonspecific β_2 -antagonism and CNS reactions. Antisense gene therapy has the potential to overcome these problems. The objective of this dissertation is to develop an antisense oligonucleotide specifically against β_1 -AR (β_1 -AS-ODN) and deliver it systemically by nonviral vector cationic liposomes. Its antihypertensive effects and underlying mechanisms will be studied in an animal model of human essential hypertension. The potential benefits on inhibiting hypertension-associated left ventricular hypertrophy and protecting the heart from myocardial infarction will be explored. The safety profile of chronic treatment with β_1 -AS-ODN will be established.

AIM 1: To develop a β_1 -AS-ODN that can inhibit β_1 -AR expression specifically and effectively without affecting other receptor subtypes.

AIM 2: To achieve efficient systemic delivery of β_1 -AS-ODN by cationic liposomes. A commercially available liposome formula with established efficacy and safety, DOTAP/DOPE, will be used to improve antisense delivery *in vivo*.

AIM 3: To test the antihypertensive effects of β_1 -AS-ODN in spontaneously hypertensive rats (SHR). BP and HR will be measured by indirect and direct methods, i.e. tailcuff and radiotelemetry.

AIM 4: To elucidate the mechanisms by which β_1 -AS-ODN reduces hypertension. The effects of antisense on cardiac output, peripheral renin-angiotensin system and CNS will be studied.

AIM 5: To investigate the effects of repeated administrations of β_1 -AS-ODN on high BP and left ventricular hypertrophy of SHR. Three repeated injections will be given to adult SHRs with established hypertension.

AIM 6: To study the safety profile of β_1 -AS-ODN after long-term treatment.

AIM 7: To explore the protective effects of β_1 -AS-ODN in an animal model of myocardial infarction. Four days after antisense treatment, rat hearts will be isolated and subjected to 30 min global ischemia followed by 30 min reperfusion.

CHAPTER 2 GENERAL METHODOLOGY

2.1 Biochemical Methods

Cell Culture

C6-2B cells and β 1-15-5B cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2.5 μ g/ml amphotericin-B at 37°C with 5% CO₂ for 3-4 days until cells grew to 80% confluency. The medium was changed to OPTI-MEM I (Life Technologies, reduced serum medium). β 1-AS-ODN or saline (as control) was added to the medium.

Preparation of Liposomes and ODN/liposome Complex

The cationic lipid 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) was mixed with the helper lipid L- α dioleoyl phosphatidylethanolamine (DOPE, Avanti Polar Lipids) at 1:1 mole ratio. The mixture was evaporated to dryness with a rotary evaporator at 30°C for 10 min. The lipid film was further dried by nitrogen for 10 min to evaporate residual chloroform and resuspended in sterile water to a concentration of 5 mg DOTAP/ml. The resultant mixture was shaken in a water bath at 30-40°C for 30 min and stored at 4°C overnight. The

suspension was then briefly sonicated, stored at 4°C and used within 3 months (175). The average diameter of DOTAP/DOPE was 170nm.

ODN/liposome complex was prepared on the day of use by mixing desired amount of ODNs with DOTAP/DOPE to the final DNA concentration of 300µg/ml in 5% (w/v) dextrose in water. The mixture was incubated at room temperature for 40-60 min to facilitate the maturation of the complex. Rats were anesthetized by inhalation of 6% isofluorene in a drop jar and injected intravenously through tongue vein.

Plasma Renin Activity and Plasma Ang II Levels

Plasma renin activity (PRA) was determined using angiotensin I radioimmunoassay kit (DuPont). Plasma Ang II levels were measured by radioimmunoassay as previously described (176).

Determination of MDA Levels in the Myocardium

Malondialdehyde (MDA) levels in the myocardium were measured in duplicate by a modified method of Ohkawa (177). Briefly, the ventricular tissues were homogenized. The reaction mixture consisted of 0.1 ml tissue homogenate, 0.4 ml 0.9% NaCl, 0.5 ml 3% sodium dodecylsulfate and 3 ml thiobarbituric acid reagent (containing equal amount of 0.8% aqueous thiobarbituric acid and acetic acid) and was heated for 75 min at 95°C. Thereafter the mixture was cooled by addition of 1 ml cold 0.9% NaCl and extracted with 5 ml n-butanol. After centrifugation at 3,000 rpm for 15 min, the butanol phase was assayed spectrophotometrically at 532 nm. Tetramethoxypropane (in amounts of 0, 0.1,

0.2, 0.4, 0.8, 1.0 nmole) served as external standard. MDA levels were expressed as $\mu\text{mole/g}$ tissue.

2.2 Molecular Biology Methods

Antisense Design

AS-ODN were designed according to the basic principles (178), synthesized and modified by backbone phosphorothioation (Gemini).

RT-PCR

At different time points after the single injection of β_1 -AS-ODN or inverted-ODN (as control), rats were sacrificed, renal cortex was dissected from left kidneys, dipped into RNAlater tissue storage buffer (Ambion) immediately and stored at -20°C . Total RNA was extracted using RNAwiz reagent (Ambion) and quantified by spectrophotometer. RNA samples from 4-5 rats of each time point were pooled. $5\mu\text{g}$ RNA was digested by DNase I, reverse transcribed by Superscript reverse transcriptase (Life Technologies) at 42°C for 50 min, and 1/20 of RT product was used to run PCR for 20 cycles. PCR primers for renin were 5'-AGGCAGTGACCCTCAACATTACCAG-3' (forward) and 5'-CCAGTATGCACAGGTCATCGTTCCT-3' (reverse) and yielded products of 362 bp. Primers for GAPDH were 5'-ATCAAATGGGGTGATGCTGGTGCTG-3' (forward) and 5'-CAGGTTTCTCCAGGCGGCATGTCAG-3' (reverse) and yielded products of 505 bp (179).

Southern Blot

RT-PCR products were subjected to Southern blotting, hybridized with psoralen-biotin labeled cDNA probes and detected using nonisotopic kits (Ambion). After exposing membranes to X-ray films, the intensity of renin mRNAs was quantified by densitometry and normalized with GAPDH mRNA levels. The experiments were repeated twice.

2.3 Pharmacological Methods

Membrane Preparation and β -AR Binding Assay

Membrane preparation and binding assay were carried out as previously described (180). Briefly, cells were detached and scraped from the cell culture plates. Rats were decapitated, and hearts and kidneys were removed. Atria, valves and fat were dissected from the ventricles. Cells and tissues were homogenized in ice-cold 50mM Tris-HCl buffer at pH 7.5. Then the suspensions were centrifuged at 48,000 $\times$ g for 10 min at 4°C. The pellet was resuspended in ice-cold 50mM Tris-HCl at pH 7.5 with 5mM MgCl₂, passed through a mosquito net to remove large pieces of connective tissues and centrifuged at 48,000 $\times$ g for 10 min. The resulting pellet was washed once more and diluted in 50mM Tris-HCl, 5mM MgCl₂, pH 7.5. Protein content was determined by Lowry's method. For saturation binding experiments, 100 μ g membrane protein was incubated with six concentrations of ¹²⁵I-(-)iodocyano-pindolol (I-CYP, NEN Life Science, 6.25-100pM) in a total volume of 250 μ l containing 50mM Tris-HCl (pH7.4), 5mM MgCl₂ at 36°C for 60 min. For the single point binding, assay was performed with

100pM ^{125}I -CYP. The nonspecific and β_2 -AR binding levels were determined in the presence of 1 μM ($\pm$)-alprenolol and 150nM CGP20712A (RBI), respectively. Then the reaction mixture was passed through Whatman GF/B glass fiber filter using Brandel harvester and the bound radioactivity was counted for 1 min. All binding assays were performed in triplicate, with the results varying by less than 10%.

Tissue Preparation and Quantitative Autoradiography

Rats were anesthetized and perfused with 0.9% saline before euthanasia. Tissues were removed and frozen in dry ice. Coronal sections of brain, horizontal sections of heart and sagittal sections of kidney (20 μm) were cut on a cryostat (Microm) at -20°C and mounted on microscope slides. Every seventh slide was stained with hematoxylin and eosin for histology. Autoradiography was performed according to the previously published protocol (181). Tissue sections were preincubated in Krebs buffer (NaCl 118.4mM, KCl 4.7 mM, MgSO_4 1.2mM, CaCl_2 1.27mM, NaH_2PO_4 10.0mM, pH 7.1) containing 0.1mM guanosine triphosphate (GTP), 0.1mM ascorbic acid and 10 μM phenylmethylsulfonylfluoride (PMSF) for 30 min at 25°C. Sections were then incubated in Krebs buffer containing 0.1mM ascorbic acid and 10 μM PMSF with 100pM I-CYP at 25°C for 150 min, in the presence of 1 μM (-)propranolol, 100nM ICI118,551 (β_2 -selective antagonist) or 100nM CGP20712A (β_1 -selective antagonist) to distinguish non-specific, β_1 - and β_2 -bindings. Labeled sections were rinsed in the same buffer, followed by two 15-min washes at 37°C in the buffer and rinsed in distilled water at 25°C. Dried sections were then exposed to X-ray films. The images were

quantitated with a computerized image analysis system (MCID, Imaging Research) and normalized using ^{125}I -standards. Non-specific binding was less than 10% of total binding.

2.4 Physiological Methods

Blood Collection

Blood samples were collected from the tail. Serum was obtained by collecting blood with no anticoagulant, incubating on ice for 1 hr followed by centrifugation at 4000rpm for 15 min. Plasma was obtained by collecting blood in tubes containing EDTA as the anticoagulant and centrifuged at 4000rpm for 15 min immediately.

Animal Surgery

Male SHR_s (250-350g, Harlan) and Sprague-Dawley rats (200-250g, Harlan) were kept in cages in a room with a 12-hr light-dark cycle. Animals were fed standard laboratory rat chow and tap water ad libitum. All care and surgical conditions were approved by the UF Animal Care Committee.

Telemetric Sensor Implantation. Before implantation, the zero of each radiotransmitter (TA11PA-C40, Data Sciences) was verified to be ≤ 4 mmHg. SHR were anesthetized with 100mg/kg ketamine and 15mg/kg xylazine and a midline abdominal incision was made. A fluid-filled sensor catheter was then inserted into the right femoral artery and the tip of the catheter was in the abdominal aorta caudal to the renal arteries. The sensor battery was sewed to

the skin in the peritoneal cavity. The implanted rats were allowed to recover for 1 week.

Jugular Vein Cannulation. One week after telemetric implantation, rats were anesthetized and a curved catheter made of PE 50 and vinyl tubing was inserted into the jugular vein. The tubing was led under the skin of the neck and exposed on the back to allow for drug infusion. Rats were allowed to recover for 24 hrs before experimentation. The catheters were flushed with 100U heparin everyday to prevent clogging.

Determination of Effects of β_1 -AS and Atenolol on Cardiovascular Parameters in Response to β -stimulation

Langendorff Heart Perfusion. 48hrs after injection of 1mg/kg β_1 -AS-ODN (n=9) or inverted-ODN (n=6), SHR were anesthetized and sacrificed. Heart perfusion was performed according to the protocol described previously (182). Hearts were quickly removed and perfused via the aorta with oxygenated Krebs buffer (118mM NaCl, 18.75mM NaHCO₃, 1.2mM KH₂PO₄, 4.7mM KCl, 1.2mM MgSO₄, 1.25mM CaCl₂, 11.1mM Glucose, 0.01mM EDTA) at a constant flow of 7.0ml/min at 36°C. Coronary perfusion pressure (CPP) was measured via a catheter placed proximal to the aorta and connected to a pressure transducer (Gould Statham P23ID). A latex balloon filled with water and connected to the pressure transducer was inserted into the left ventricle through the left atrium to measure left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), and developed left ventricular pressure (dLVP) (dLVP=LVSP-LVEDP). LVEDP during equilibration was set at 5 to 7mmHg. CPP, LVEDP and

LVSP were continuously recorded on a 4-channel recorder (Astro-Med). After baselines for dLVP and heart rate (HR) were stable for 5 min, isoproterenol (ISO, non-specific β -agonist) was given at 0.01, 0.025, 0.05, 0.12 μ M at 10 min intervals so as to avoid the effect of tachyphylaxis.

Telemetric Monitoring of Live Animals. The effects of β_1 -AS-ODN and atenolol on cardiac $dP/dt_{\max}$, HR and systolic BP (SBP) were compared in the same group of SHR (n=4). Two days after catheterization of jugular vein, control values were taken and 1mg/kg β_1 -AS-ODN was injected. 48 hrs later, rats were tested for the effect of β_1 -AS-ODN. The rats were allowed to recover until all the cardiovascular parameters returned to control values. Then 1mg/kg atenolol (β_1 -selective antagonist) was injected and rats were tested 30 min later. For β_1 -stimulation, SHR were infused with dobutamine (β_1 -selective agonist) through jugular vein catheter at 5, 10, 20, 40 μ g/kg/min. Each dose was given for 5 min continuously and at 1 hr intervals until all the cardiovascular parameters returned to baseline so as to avoid the effect of tachyphylaxis. BP and HR were sampled every 1 min. $dP/dt_{\max}$ was calculated from the slope of the rising pulse pressure curve and determined every 1 min. The difference between values at each dose and baseline was denoted as Δ .

BP and HR Monitoring

Telemetry. Each rat cage was placed on a receiver (RLA1020, Data Sciences) for measurement of cardiovascular parameters. Data were collected with a computer-based data acquisition program (Dataquest LabPRO3.0; Data

Sciences). BP and HR were measured every 10 min and averaged every 1-24 hrs. Before treatment, SHR were monitored for a week to get a stable baseline.

Tailcuff. Rats were warmed for 20-30 min in cages on heating pads. The temperature was controlled at 35-37°C. Then rats were placed in a plastic restrainer kept at 37°C. A pneumatic pulse sensor was attached to the tail. After cuff inflation, systolic BP was determined as the first pulsatile oscillation on the descending side of pressure curve. Heart rate (HR) was determined by manual counting of pulse numbers per unit time. BP and HR were recorded by a Narco physiograph. Data values of each rat were taken as an average of at least four stable readings. Baseline was determined by averaging three days of measurements before antisense administration.

Myocardial Ischemia-reperfusion

SD rats were anesthetized with sodium pentobarbital (40 mg/kg) intraperitoneally. The hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer (118 mM NaCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 4.7 mM KCl, 1.2 mM MgSO₄, 1.25 mM CaCl₂, 11 mM Glucose, pH 7.4). Within 1 min, the hearts were transferred to an Langendorff apparatus and perfused via the aorta with oxygenated (95% O₂ + 5% CO₂) Krebs-Henseleit buffer at 37°C using a MasterFlex pump (model 7015-21, Cole-Palmer Instrument). The heart was placed in a semiclosed circulating water-warmed (37°C) air chamber, paced atrially at a rate of 300 bpm with a Medtronic 530 pacemaker, and perfused at a constant flow (5.5-6.0 ml/min). LVEDP during equilibration was set at 5-7 mmHg.

Sham controls were continuously perfused with Krebs-Henseleit buffer for 80 min. Heart from other groups, were equilibrated for 20 min, subjected to 30 min global ischemia followed by 30 min reperfusion. After completion of the experiment, hearts were frozen in liquid nitrogen for β -AR radioligand binding assay and measurement of MDA.

2.5 Clinical Pathology Methods

Liver Transaminase Levels

The plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by a colorimetric method using a kit (Sigma, Cat. #505-OP). Briefly, plasma samples were incubated with proper substrates for ALT or AST at 37°C for 1 hr and subjected to color reactions. The absorbency was read at the wavelength of 500 nm and the concentrations of ALT and AST were calculated from the calibration curve obtained under the same conditions.

Hematology

Hematology measurements were carried out in the Clinical Laboratory of Shands Hospital at University of Florida. The hematology parameters tested included erythrocyte count (RBC), hematocrit, platelet count and total leukocyte count (WBC).

C-Reactive Protein (CRP)

CRP values were obtained on blood serum in the Clinical Laboratory of Shands Hospital at University of Florida.

Histopathology

Rats were euthanized by a peritoneal injection of overdose pentobarbital. Selected tissues, including heart, liver, spleen and kidney, were removed, trimmed of fat and contiguous tissues and weighed. These tissues were fixed in 10% neutral buffered formalin, embedded in paraffin (Diagnostic Referral Laboratory, University of Florida), sectioned at the thickness of 5 μ m, mounted on gelatin-coated slides, stained with hematoxylin and eosin, and examined under light microscopy.

Gel Immunodiffusion Assay

Specific antibodies to ODN/liposome complex were measured with double immunodiffusion (Ouchterlony) method on premade discs purchase from ICN Biomedicals. Briefly, the ODN/liposome complex was loaded in the center well, and serum samples in the surrounding wells. They were allowed to diffuse at room temperature in a moisturized and sealed container for up to 7 days. The occurrence of precipitation bands indicated the presence of antibody-antigen reaction.

2.6 Statistical Analysis

All experiments were repeated at least 2 times unless the situation and results called for more experiments. Various statistical test were performed depending

on the experimental design using Jandel Sigmastat Software. Values were expressed as mean $\pm$ SEM. Student *t* test was used to evaluate significant differences when two groups were used. One way analysis of variance (ANOVA) and appropriate multiple range test were used when more than two groups were compared. Pearson product moment correlation was used to assess the relationship between two parameters. In some studies, a two-way ANOVA were utilized to evaluate the effects of individual treatments and any interactions between the treatments. For analysis of parameters (e.g. dP/dt, BP, HR by telemetry) obtained from the same animal over time, repeated measure of ANOVA was incorporated. Significance for all tests was set at the 95% confidence limit. All molecular biology experiments dealing with the comparison of mRNA by RT-PCR were carried out in the linear range of the assay. The data were normalized by GAPDH and subjected to appropriate statistics.

CHAPTER 3 OPTIMIZATION OF ANTISENSE DESIGN AND DELIVERY

3.1 Introduction

Antisense oligodeoxynucleotides (AS-ODN) are generally 15-20 bases in length. Theoretically the length of 12-15 bases is able to provide sufficient specificity and binding affinity for a target mRNA (128) while short enough to gain entry into cells (183). The specificity of antisense molecules is so great that even two genes that differ by a single base mutation can be differentially inhibited (129). There is a wide range of potential sites within an mRNA sequence for antisense targeting, the best of which is considered to be in the vicinity of AUG translation initiation codon. The availability of these sites largely depends on the folding of the mRNA (184). However currently there is no computer program sophisticated enough to predict the secondary structure of the target mRNA. Lacking a sure-fire method of oligo prediction, antisense development has to go through a trial-and-error test.

The basic principles of antisense design are described below, which are now published in *Methods in Enzymology* (178).

1. *Identify the sequence:* Check GenBank for the mRNA sequence of the target protein in the species to be studied. If there is more than one lab cloning the same protein, compare the homology of different reports. These sequences will point out the controversial bases, which can be due to natural mutations

in variant strains of the same species or sequencing errors. Try to avoid these debatable regions. Target the ODN to the identical regions, which ensures reliability of the sequence being used.

2. *Target the sites:* Although there are several possible sites within the DNA sequence to target for antisense design, the regions that are considered to be the best targets for constructing effective AS-ODNs are the 5' cap region and the area around the AUG translation initiation codon. In our experience, AUG start codon and nearby bases downstream of AUG in the coding region are most promising sites for antisense inhibition (185). Usually the length of 15-20 mer is sufficient to guarantee sequence specificity, which must be confirmed by checking with GenBank for existing sequences to avoid any significant homology with other mRNAs.
3. *Modify the backbone:* AS-ODNs are chemically modified to increase nuclease resistance and stability. The most commonly adopted modification is backbone phosphorothioation, in which one non-bridging oxygen of the phosphodiester linkage is replaced by sulfur (137). Compared to natural DNA, phosphorothioated ODNs have a greatly extended half-life of 42-56 hrs in the plasma of rat, monkey and human (186-188). They are also better substrates to induce RNase H cleavage of mRNA. But phosphorothioation significantly decreases the melting temperature (T_m) of ODNs and reduces binding affinity and stability of DNA/DNA and DNA/RNA duplexes. For 15-25 mer, T_m of phosphorothioate ODN is usually 7-12°C lower than normal ODN, with AT bases showing more T_m depression than GC (189,190). This fact

must be taken into account in the design of short AS-ODN, because 15-mer phosphorothioate ODN /RNA hybrids may have T_m close to body temperature of 37°C.

4. *Avoid pitfalls:* Some other basic rules applicable in the design of polymerase chain reaction (PCR) primers also hold true for AS-ODN (191), e.g. avoidance of palindromic structure and primer-dimer formation, and choosing sequence with a balance AT/GC ratio to minimize toxicity and non-specific binding caused by high GC content. Although a high GC/AT ratio should be avoided generally, the danger of toxicity and non-specific binding can be reduced by phosphorothioation.

3.2 Results

3.2.1 Design of β_1 -AS-ODN

My plan was to develop a 15 mer β_1 -AS-ODN specific to rat (192) and human (11) β_1 -AR mRNA. Thirteen sequences were picked within the conserved regions between rat and human β_1 -AR mRNA, which covered a variety of sites including upstream of AUG, spanning AUG, CDS and 3'-UTR. Seven were discarded after specificity checking by Blast search and secondary structure analysis of the ODNs. The remaining six candidates are listed below. They are modified by backbone phosphorothioation. Their positions refer to rat β_1 -AR mRNA (gene accession number: J05561) (192).

#1: 5'- ccg cgc cca tgc cga -3' (64-78, spanning AUG start codon)

#4: 5'- ggt cgt tgt agc agc -3' (691-705, in the CDS)

- #6: 5'- cgg tct tga gta aac -3' (1602-1616, in the 3'-UTR)
- #7: 5'- ggc cga cga cag gtt -3' (111-125, in the CDS)
- #8: 5'- atg agc agc acg atg -3' (269-283, in the CDS)
- #10: 5'- ttg gtg agc gtc tgc -3' (335-349, in the CDS)

3.2.2 *In vitro* and *in vivo* Screening

In vitro

The effects of β_1 -AS-ODN on the expression of β_1 -AR were examined in C6-2B and β_1 -15-5B cell lines. C6-2B is a rat glial tumor cell line. β_1 -15-5B is a mouse connective tissue cell line NCTC 2071 permanently transfected with human β_1 -AR cDNA. Both cell lines express abundant β_1 -AR. Cells were treated with 0.1-10 μ M β_1 -AS-ODN or saline (as control) for 3-48 hrs. β_1 -AR levels on the cell membrane were measured with radioligand binding assay. Many experiments were performed testing different doses and time course, but all yielded negative results. None of the antisense candidates shown above reduced β_1 -AR levels in either cell line. A representative experiment was summarized in Table 3-1 and 3-2.

Reasons for the absence of antisense action were searched for. Although efficient uptake of 15-18 mer S-ODN in cell cultures was shown in early studies (183), the possibility still existed that AS-ODN did not enter cells. To answer this question, cells were treated with fluorescein-labeled AS-ODN and the ODN uptake was examined under confocal microscopy. 30 min after addition into the medium, fluorescence was readily observed and widely distributed in the cytoplasm of the majority of the cells. Another reason could be that cells were

not treated with an optimal dose of AS-ODN for enough time. Therefore, experiments on dose response (0.1 μ M, 1 μ M, 2 μ M, 5 μ M, 10 μ M) and time course (3hr, 6hr, 12hr, 24hr, 48hr) were performed and repeated, but still showing no significant effects of AS-ODN on β_1 -AR expression. It was noticed that high doses (5 μ M, 10 μ M) of phosphorothioate ODN seemed to be toxic because cell viability and total protein content per plate were significantly reduced compared to saline control in some experiments. More experiments were planned, including liposomal delivery of AS-ODN and effects of AS-ODN on cellular responses to β_1 -agonists. However, despite continuous efforts, β_1 -AS-ODN failed to reduce β_1 -AR levels *in vitro*.

Table 3-1. Effects of β_1 -AS-ODN on C6-2B cells.

C6-2B Cell	Cell count /plate	total protein /plate (mg)	β -AR binding (fmol/mg)	β -AR binding (% of Control)
Saline	5.4×10^6	0.71	57	100
β_1 AS 1	4.4×10^6	0.56	68	119
β_1 AS 7	4.8×10^6	0.80	48	84
β_1 AS 8	5.2×10^6	0.54	53	93
β_1 AS 10	4×10^6	0.60	54	95

Cells were grown in DMEM / 5%FBS until 80% confluency and the medium was changed to OPTI-MEM I (GIBCO BRL, reduced serum medium). 5 μ M β_1 -AS-ODN or saline were added to the medium 24hr before a single point binding assay on membrane proteins at 100 pM ^{125} I-CYP. Non-specific binding was obtained in the presence of 1 μ M alprenolol.

Table 3-2. Effects of β_1 -AS-ODN on β_1 -15-5B Cells.

β_1 -15-5B Cell	Cell count /plate	total protein/plate (mg)	β -AR binding (fmol/mg)	β -AR binding (% of Control)
Saline	5.2×10^6	0.53	156	100
β_1 AS 1	5.2×10^6	0.50	175	113
β_1 AS 7	5.0×10^6	0.49	151	97
β_1 AS 8	4.8×10^6	0.49	165	106
β_1 AS 10	4.8×10^6	0.50	163	104

In vivo

It was hypothesized that an effective β_1 -AS-ODN, by suppressing the β_1 -AR expression and function in heart and kidney, could efficiently inhibit hypertension. Hence, the effectiveness of β_1 -AS-ODN was evaluated based on the extent of reduction of β_1 -AR expression and high BP in rats. To facilitate the antisense delivery, ODNs were complexed by cationic liposomes DOTAP/DOPE at a (+/-) charge ratio of 0.5.

Antisense efficacy was first tested in normotensive Sprague-Dawley (SD) rats, which received a single intravenous injection of β_1 -AS-ODN through tongue vein. The inhibition of β_1 -AR levels in the heart was assessed by saturation receptor binding assay two days after treatment. All antisense candidates significantly reduced the cardiac β -AR levels to different extents as compared with saline or inverted-ODN (INV-ODN) control (Table 3-3). Based on the receptor level, ODN-1 and ODN-7 were better than other candidates.

Table 3-3. Effects of β_1 -AS-ODNs on cardiac β -AR in rats.

Treatment	R	KD (pM)	Bmax (fmol/mg)	Decrease in percentage
Saline (n=3)	0.9716	55 $\pm$ 3	42.1 $\pm$ 1.6	
INV-ODN-1 (n=3)	0.9217	56 $\pm$ 2	38.7 $\pm$ 1.2	
AS-ODN-1 (n=3)	0.8650	34 $\pm$ 1	27.5 $\pm$ 0.8*	31%
AS-ODN-7 (n=3)	0.9500	32 $\pm$ 2	26.0 $\pm$ 1.5*	35%
AS-ODN-8 (n=3)	0.9712	36 $\pm$ 3	28.9 $\pm$ 2.1*	27%

Male SD rats (250-300g) were treated with a single i.v. injection of 200 μ g β_1 -AS-ODNs or INV-ODN for 48 hrs. Membrane proteins were prepared from heart ventricles. Affinity and density of β -AR were measured by radioligand binding assay. $P < 0.01$ versus saline or INV-ODN.

The antihypertensive effects of β_1 -AS-ODNs were then tested in spontaneously hypertensive rats (SHR) monitored by radiotelemetry. A single injection of ODN-1 significantly diminished mean arterial pressure by up to 14 mmHg for 8 days ($P < 0.05$), whereas the drop in BP produced by ODN-7 was less marked (8 mmHg) and for a shorter duration (4 days). The hypotensive impact of β_1 -AS-ODN was dose-dependent, with the order 800 μ g > 400 μ g > 200 μ g.

Based on these results, ODN-1 was determined to be the optimal antisense sequence for β_1 -AR which reduced β_1 -AR expression and high BP most effectively.

3.2.3 Optimization of Antisense Delivery by Cationic Liposomes

Although chemical modifications like phosphorothioation endow AS-ODN with nuclease insensitivity and longer half-life, the stability and delivery of AS-ODN must be further improved to be effective *in vivo*. Cationic liposomes are known for their ability to facilitate cellular uptake of nucleic acids and protect them from degradation in circulation and tissues (165,169). Numerous lipid formulations have been developed to enhance the transfection of plasmid DNA and oligonucleotides (168,193). Dioleoyl trimethyl ammonium propane (DOTAP) was introduced in 1980s and remains one of today's favorite lipids for its effectiveness and biodegradability (163,194,195). Not only has its usefulness been proven in bench studies but also explored in clinical trials, e.g. to deliver CFTR gene for cystic fibrosis. Therefore we chose to use DOTAP coupled with the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) to deliver β_1 -AS-ODN in our study.

However, high transfection efficiency that was seen *in vitro* could not be readily achieved *in vivo* with the same liposome/DNA preparation. This is in part attributed to the serum resistance of liposome/DNA complex because negatively charged plasma proteins can bind to cationic lipids and destabilize liposome/DNA complex (196). It was recently shown that increasing the +/- charge ratio of liposomes to DNA and inducing the maturation of liposome-DNA complex by prolonging incubation time could overcome this problem (169,197,198). The optimal charge ratio of DOTAP:DNA was found to be ≈ 2.0

(198,199). Thus I chose to test five charge ratios ranging from 0 to 3.5 to optimize the delivery of β_1 -AS-ODN. In addition, DOTAP/DOPE and ODN were carefully mixed and incubated at room temperature for 40-60 min before use to facilitate stabilization of the complex.

Figure 3-1 shows the effect of different (+/-) liposome:ODN charge ratios on systolic BP of SHR measured by tail cuff (n=6 for each ratio) in a representative experiment. 0.5 mg/kg β_1 -AS-ODN alone, i.e. at ratio 0, did not change SBP, whereas ratio 0.5 significantly reduced SBP by up to 38 ± 5 mmHg for 7-8 days. When the ratio was increased, the duration of the hypotensive impact was drastically prolonged to 18-20 days at ratio 1.5, 20-33 days at ratio 2.5 and 3.5, varying with liposome preparations. However the maximum drop in SBP was greater at ratio 1.5 and 2.5 (~ 35 mmHg) than at ratio 3.5 (~ 25 mmHg) (Table 3-4). Accordingly, the optimal charge ratio of DOTAP:ODN was determined as 2.5 and employed in the subsequent experiments. It was worth noting that the antihypertensive effect of β_1 -AS-ODN varied slightly with different batches of liposome mixture, probably due to the variation in particle size and structure. Furthermore, aggregation and precipitation of liposome/ODN must be avoided to ensure the successful delivery.

Table 3-4. Amplitude and duration of reduction in blood pressure of SHR after a single i.v. injection of 0.5 mg/kg β_1 -AS-ODN delivered at different charge ratios of liposome/ODN.

Charge ratio of liposome/ODN	Maximum reduction in blood pressure (mmHg)	Range of reduction (mmHg)	Duration (day)
0	2	--	--
0.5	35-38	24-38	7-8
1.5	28-35	18-35	18-20
2.5	30-34	20-34	20-33
3.5	20-24	15-24	20-33

3.3 Discussion

The purpose of this study is to develop an effective β_1 -AS-ODN targeted to rat and human β_1 -AR mRNA that can reduce β_1 -AR expression and BP in hypertensive models. After *in vitro* and *in vivo* screening, the best β_1 -AS-ODN candidate was selected which was 15 mer in length and spanned the AUG site. It significantly reduced the β_1 -AR levels in the heart and lowered high BP in SHR. The delivery of AS-ODN was improved by cationic liposomes DOTAP/DOPE and the condition was optimized.

These results support the notion that the region around the translation initiation codon may be the best target for antisense design. ODN binding to this area may interfere with the ribosomal assembly and/or form a structural hurdle to ribosome sliding along the mRNA strand. Assuming that all the six β_1 -AS-ODN candidates can bind to β_1 -AR mRNA, it is conceivable that interrupting the initial

phase of translation may be more effective than later blockade. However, this assertion is more or less arbitrary, because I do not have evidence to show that all candidates have access to β_1 -AR mRNA at the same affinity. In spite of this, since it is unlikely to screen every possible region in an mRNA for economical reasons, I believe the first site to consider for AS targeting is the vicinity of AUG codon if other criteria can be met.

Efficient gene delivery is vital to the therapeutic application of AS-ODN *in vivo*. Among nonviral vectors, cationic liposomes are most widely used. They are safe, nonimmunogenic and easy to produce on a large scale. However, relatively low transfection efficiency has been obtained after intravenous administration, mainly due to the inactivation of cationic liposome by serum. It was shown recently that increasing the charge ratio (+/-) of liposome to DNA and inducing the maturation of liposome-DNA complex can overcome this problem (197,198). The optimal charge ratio of DOTAP:DNA was demonstrated to be ≈ 2 (198,199). Based on these studies, the delivery of AS-ODN was optimized by testing five charge ratios from 0-3.5. As shown in the results, increasing the charge ratio not only improved the delivery efficiency but prolonged the duration of β_1 -AS-ODN action. The best antihypertensive result (35 mmHg for 20-33 days) was achieved at ratio 2.5 consistently.

The prolonged effect of β_1 -AS-ODN on BP may be attributed to several reasons. The half-life of phosphorothioate ODN is ~ 50 hrs in rat plasma after intravenous injection. Li *et al.* (183) showed that S-ODN could be found in its intact form for at least 5 days after entry into cells. It has been shown that

liposome encapsulation not only improves the cellular uptake of DNA, but also protects DNA from degradation and extends its circulation time *in vivo* (165,169). In addition, Baker's group (180,200) and other researchers (201) have shown that β -ARs had a slow turnover rate which took 200-250 hrs to recover in the heart after irreversible blockade.

Then a question arose, i.e. why β_1 -AS-ODN reduced β_1 -AR density on the cell membrane so rapidly (within 2 days), provided that β -ARs had a slow turnover rate. It is possible that β_1 -AS-ODN not only inhibited the translation of β_1 -AR, but also decreased the intracellular pool of β_1 -AR and accelerated the internalization of the membrane receptors. The recycling of receptors between cell membrane and cytoplasm is a much faster process than receptor metabolism. This may explain the discrepancy between the rapid reduction in β_1 -AR density and the slow turnover. However to test this hypothesis requires further experiments.

It is still not understood why β_1 -AS-ODN did not decrease β_1 -AR levels *in vitro*. Similar protocols had been successfully used in our lab to screen for AS-ODN against AT1-receptor in cell culture. The possibility that AS-ODN failed to enter cells was ruled out by the visualization of fluorescein-labeled AS-ODN in the cytoplasm 30 min after addition into the medium. Further experiments on dose response and time course did not improve the effects. We stopped *in vitro* study after 6 months of pursuing. However, it is possible that AS-ODN delivery in cell culture could have been improved by liposomes, as the case *in vivo*. It is also

likely that AS-ODN, after the endocytosis, did not escape endosomes to interact with target mRNA.

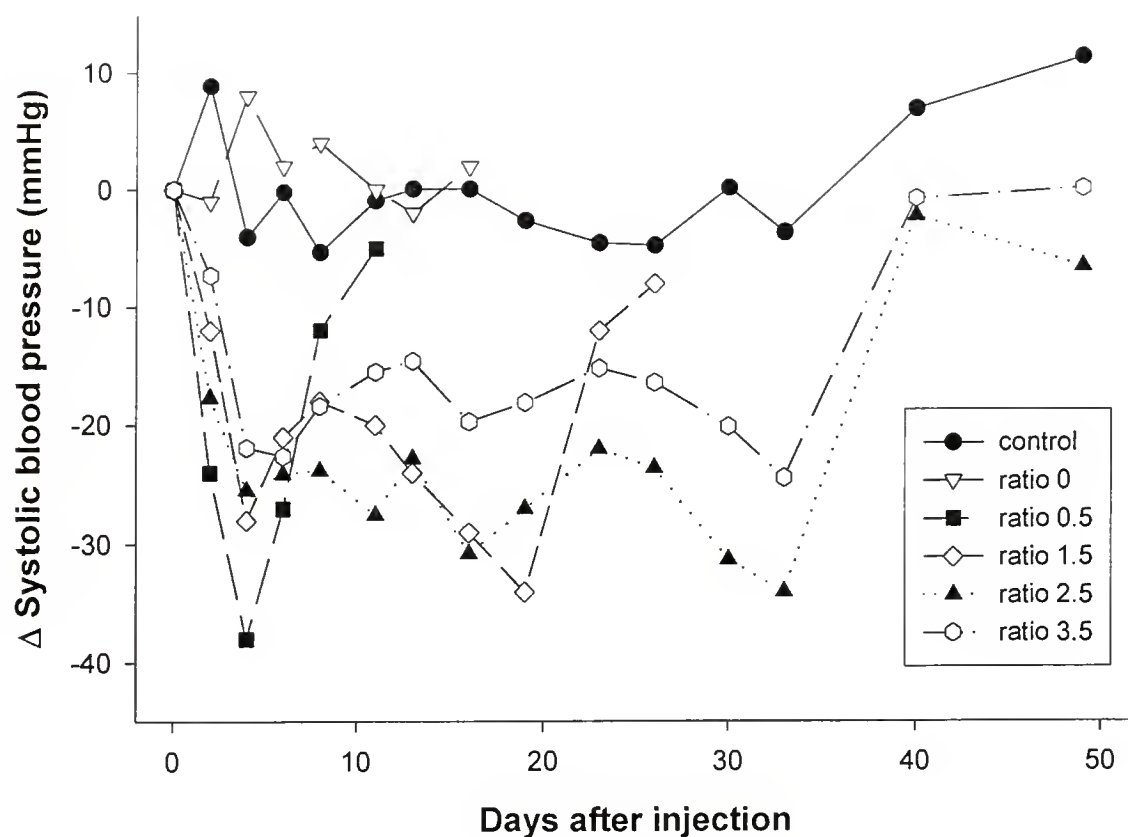


Figure 3-1. Improving the antihypertensive effect of β_1 -AS-ODN by optimization of liposome:ODN charge ratios.

SHR received a single iv injection of β_1 -AS-ODN or INV-ODN. 1mg/kg β_1 -AS-ODN was delivered by DOTAP/DOPE at charge ratios from 0 to 3.5. 1mg/kg INV-ODN delivered by DOTAP/DOPE at charge ratio 2.0 served as control (●). Data represent mean values of each group (n=6). Standard errors were omitted for clarity.

CHAPTER 4

EFFECTS ON BLOOD PRESSURE AND HEART RATE: β_1 -AS-ODN vs. ATENOLOL

4.1 Introduction

β_1 -AR plays a crucial role in the regulation and maintenance of hypertension. β_1 -adrenergic activation increases cardiac contractility and heart rate, and stimulates renin production from the kidney. Thus the inhibition of this receptor can lead to decreased cardiac output and renin-angiotensin activity and thereby reduce hypertension. This concept has been proven correct by the antihypertensive action of β -blockers in clinical practice. In developing an antisense agent for β_1 -inhibition to reduce hypertension, the rationale borrows from the success of β -blockers because both approaches can suppress β_1 -AR and therefore β_1 -AS should be able to lower high BP if β -blockers do. However these two strategies adopt different pathways for β_1 -inhibition. Antisense reduces the total numbers of β_1 -AR by interfering with translation, while β -blockers dampen the function of β -receptors by competing with endogenous β -agonists like norepinephrine. Due to the intrinsic drawback of pharmacological intervention, β_1 -specific antagonists have not been developed which inevitably results in undesirable antagonism of β_2 -receptor subtype. This accounts for a large portion of side effects caused by β -blockers including contraindication of asthma, peripheral vascular disease and insulin-dependent diabetes mellitus,

and reduced exercise capacity. In contrast, antisense can be designed specific to its target based on gene sequence. This will preclude adverse effects of nonspecific antagonism by β -blockers. Furthermore, CNS reaction can be avoided by antisense owing to the inability of antisense molecules to enter the brain. Here the effects of β_1 -AS-ODN on BP and HR of spontaneously hypertensive rats (SHR) were tested and compared with those of a hydrophilic β_1 -selective blocker, atenolol.

4.2 Results

4.2.1 Effects of β_1 -AS-ODN on BP and HR Measured by Tail cuff

Figure 4-1 shows a typical experiment in which SHR was treated with a single i.v. injection of 1mg/kg β_1 -AS-ODN delivered by the optimal ratio of cationic liposomes determined in Chapter 3. BP and HR were measured by the traditional tail cuff method. Antisense significantly decreased systolic BP for 4 weeks. The maximum drop was 30 mmHg. However, HR was not changed by β_1 -AS-ODN. Inverted-ODN (INV-ODN) as control had no effect on BP or HR.

4.2.2 Effects of β_1 -AS-ODN on BP and HR Measured by Radiotelemetry

In order to compare the effects of β_1 -AS-ODN and atenolol, radiotelemetry was used to monitor BP and HR on a regular basis. Radiotelemetry allows for accurate and continuous hemodynamic monitoring without inducing stress or artificial factors. 1mg/kg β_1 -AS-ODN delivered with liposomes at the (+/-) ratio 0.5 produced a maximum drop of 15mmHg in mean blood pressure (Fig. 4-2).

The antihypertensive effect lasted for 8 days, which was consistent with the results measured with tailcuff. HR was not significantly altered. In contrast to AS-ODN, although the onset of hypotensive effect caused by 1mg/kg atenolol occurred as early as 20 min after injection, it lasted for only 10 hrs (Fig. 4-3). In addition, atenolol caused considerable bradycardia up to an average of -75 bpm. All these hemodynamic changes with i.v. atenolol were not detectable within a day.

4.3 Discussion

The results indicate that the antihypertensive effect of β_1 -AS-ODN is significantly longer lasting than that of atenolol. A single iv injection can reduce BP for 20-30 days with the maximum drop of 30 mmHg. In contrast, the hemodynamic response to bolus iv injection of atenolol lasts only for hours. As a result, atenolol must be taken every day to achieve chronic control of BP. Moreover, atenolol causes the peak-and-trough fluctuation in BP, in contrast to the steady hypotensive action of antisense. Greater than normal variation in 24-hr BP is potentially dangerous and associated with early morning cardiovascular catastrophe. Finally, β_1 -AS-ODN does not decrease HR as shown by both tail cuff and telemetry, whereas atenolol induces appreciable bradycardia. Bradycardia is a common complaint by patients taking β -blockers.

The most surprising finding of this study is the lack of effects on HR of β_1 -AS-ODN. The mechanisms are still under investigation. Several possibilities can be considered. First, β_1 -AS-ODN did not affect β_2 -AR, which played an important

role in the regulation of HR (202,203), while not involved in the cardiac contraction of rats. Second, antisense inhibition of β_1 -AR expression is gradual and to a lesser extent than β -blockers. It is also possible that β_1 -AR may have a larger reserve for controlling HR than contractility. However, to answer this question requires more extensive studies and electrophysiology techniques which are beyond the scope of this dissertation.

Both tailcuff and telemetry measures of BP showed a significant drop after antisense treatment. SHR responded to β_1 -AS-ODN to a greater degree of hypotension when subjected to tailcuff versus telemetry. In addition, the baseline measured with tailcuff was consistently higher than that with telemetry by 20-30mmHg in the same rats. Bazil *et al.* (204) reported a similar phenomenon. They compared the cardiovascular parameters recorded by telemetry, tailcuff and arterial catheter and observed a more sensitive hypotensive effect of captopril with tailcuff. Tailcuff measurement of BP involves warming and restraint of rats. It is conceivable that β_1 -AS, through the suppression of sympathetic activity, can decrease BP of animals under stress more effectively.

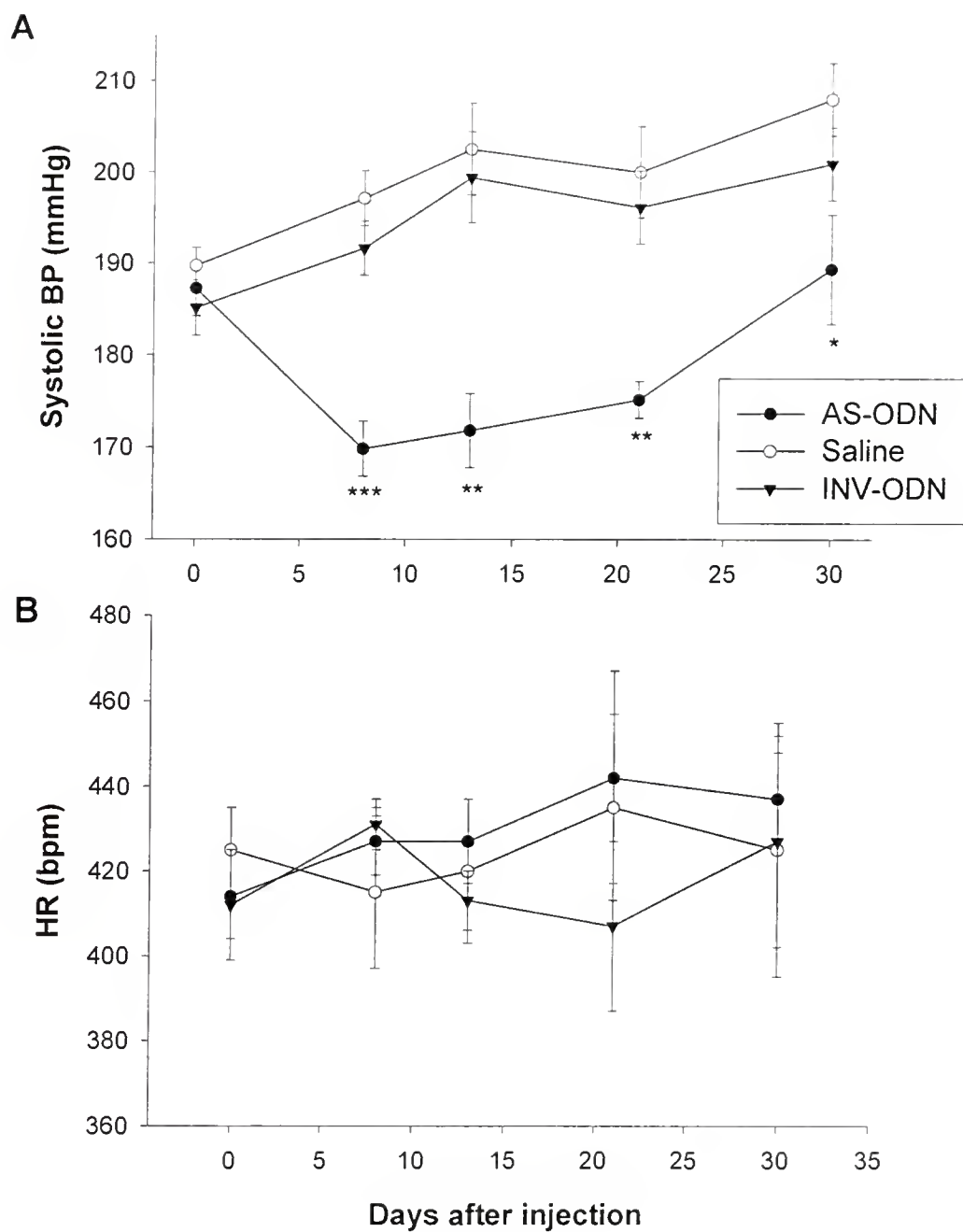


Figure 4-1. Effects of β_1 -AS-ODN on BP and HR of SHR.

16-week old SHR were treated with a single iv injection of 1mg/kg β_1 -AS-ODN delivered by DOTAP/DOPE at a (+/-) charge ratio of 2.5. BP (A) and HR (B) were measured by tail cuff method. Data represent mean $\pm$ SEM (n=6-9 for each group). *** P<0.001, ** P<0.01, * P=0.059 vs. saline or INV-ODN.

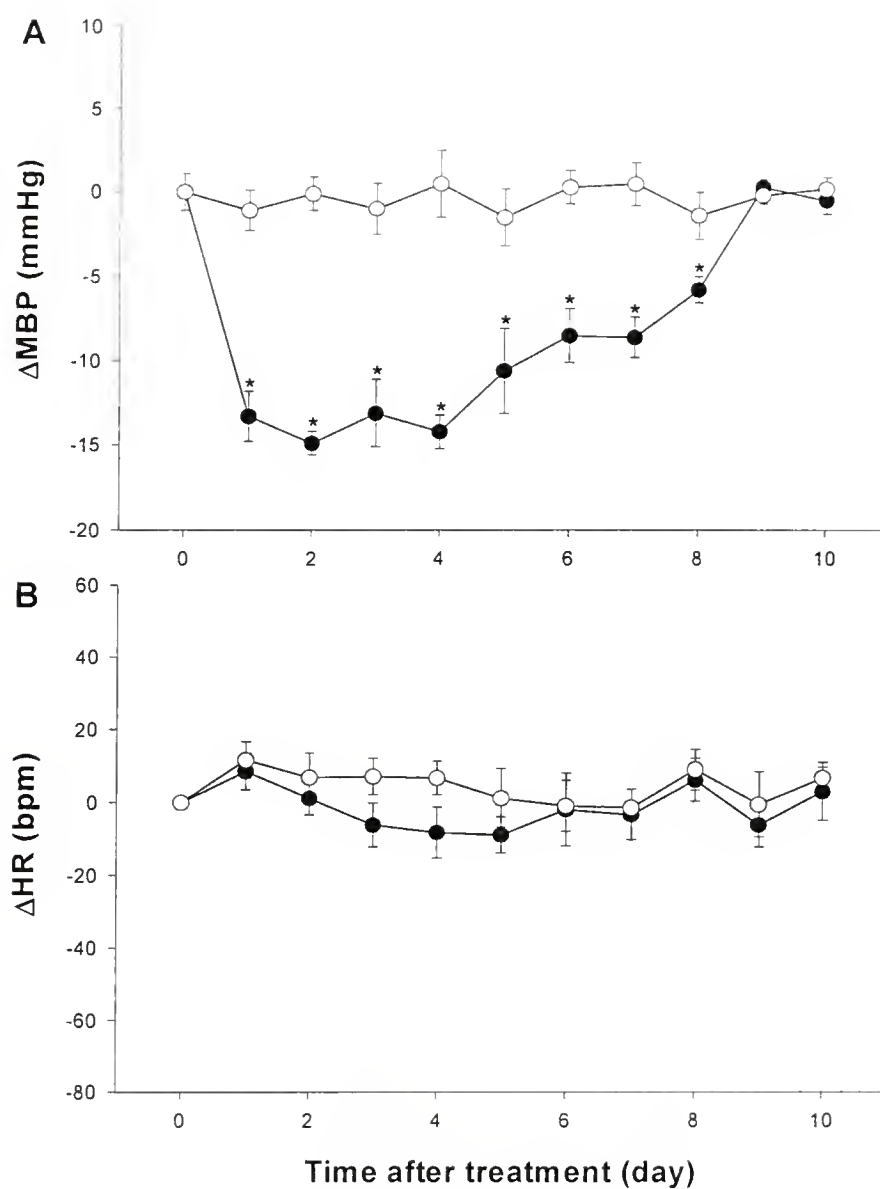


Figure 4-2. Effects of β_1 -AS-ODN on mean BP and HR of SHR monitored by telemetry.

1mg/kg INV-ODN (O) or β_1 -AS-ODN (●) was injected with liposomes at a (+/-) charge ratio 0.5. BP (A) and HR (B) were recorded every 10 min and averaged every 24 hrs. Data represent mean $\pm$ SEM of each group (n=4). * $P < 0.05$ vs. INV-ODN.

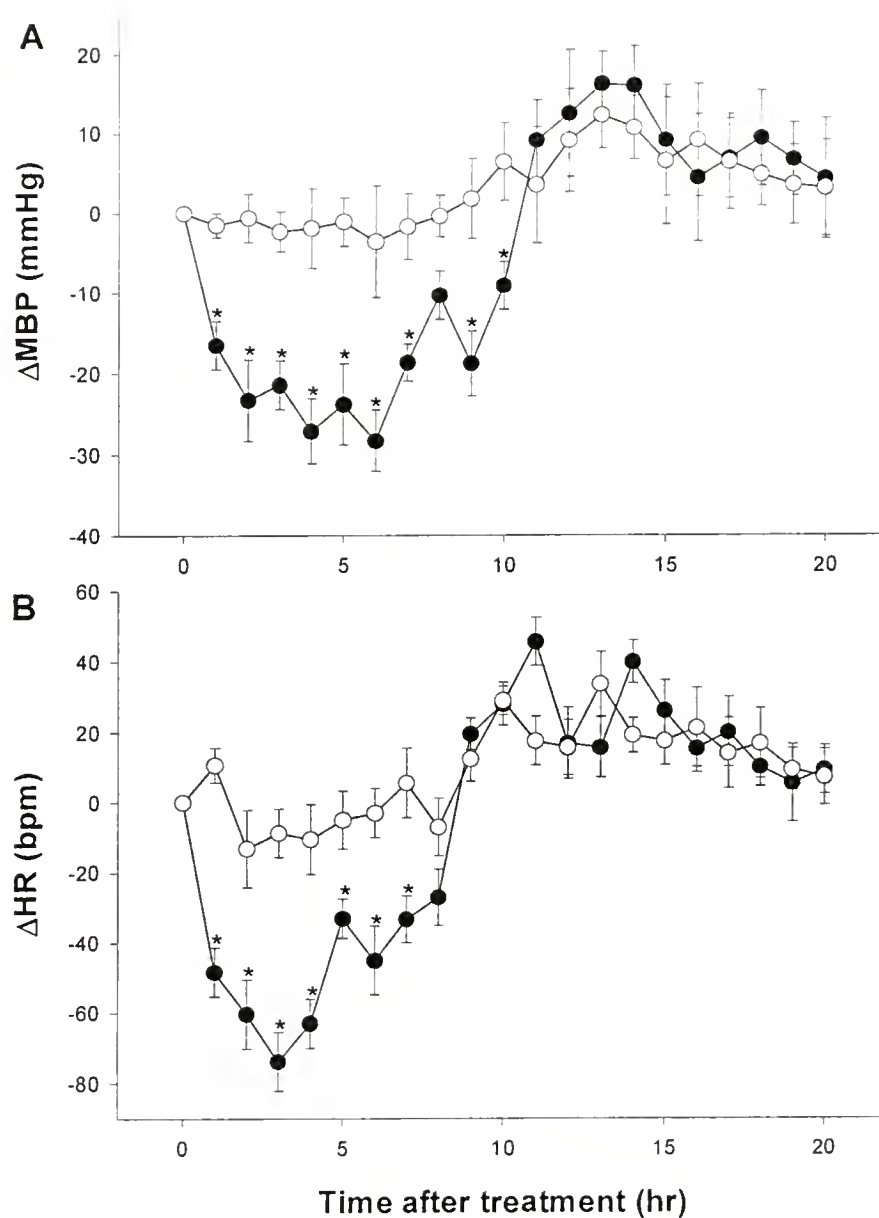


Figure 4-3. Effects of atenolol on mean BP and HR of SHR monitored by telemetry.

Saline (O) or 1mg/kg atenolol (●) was injected intravenously. BP (A) and HR (B) were taken every 10 min and averaged every 1 hr. Data represent mean $\pm$ SEM of each group (n=4). * P<0.05 vs. saline.

CHAPTER 5

MECHANISMS FOR THE ANTIHYPERTENSIVE EFFECTS OF β_1 -AS-ODN

5.1 Introduction

In the preceding studies, a β_1 -AS-ODN was developed which lowered BP of SHR effectively and persistently with a single i.v. injection. The current study is carried out to understand the mechanisms underlying its antihypertensive effects. β_1 -AR activation induces positive inotropic and chronotropic responses in the heart, and stimulates renin production and release in the kidney. β -blockers decrease cardiac output and plasma renin activity, which contribute to the BP reduction. Therefore I hypothesize that:

- i) β_1 -AS-ODN, through specific inhibition of β_1 -AR expression, will decrease the functional sensitivity of β_1 -AR-mediated responses in the face of sympathetic activation, and thereby achieve an antihypertensive effect.
- ii) Since the β_1 -AS-ODN is designed specifically to β_1 -AR, it should not affect the expression of β_2 -AR subtype.
- iii) β_1 -AS-ODN cannot cross blood-brain barrier to affect β -AR in the CNS.

5.2 Results

5.2.1 Inhibition of Cardiac Contractility

Effects on cardiac β -AR levels

Fig. 5-1 shows that a single i.v. injection of 1mg/kg β_1 -AS-ODN delivered with cationic liposomes at a (+/-) charge ratio of 2.5 significantly reduced the β_1 -AR density in the SHR hearts for 18 days ($P<0.01$). The drop was at its maximum of 47% on day 4, 33% on day 10 and maintained at 29% on day 18. In contrast, there was no significant change in the $B_{\max}$ of β_2 -AR. K_D of both subtypes remained unaltered (Table 5-1). Consequently, the β_1/β_2 subtype ratio in the ventricles was diminished from ~70/30 to ~50/50 by β_1 -AS-ODN. INV-ODN had no effect on either subtype.

Table 5-1. Effects of β_1 -AS-ODN on $B_{\max}$ and K_D of β_1 - and β_2 -AR in cardiac ventricles of SHR.

	Total ($\beta_1+\beta_2$)		β_1		β_2	
	K_D (pM)	$B_{\max}$ (fmol/mg)	K_D (pM)	$B_{\max}$ (fmol/mg)	K_D (pM)	$B_{\max}$ (fmol/mg)
Saline	55.4 $\pm$ 6.7	27.6 $\pm$ 0.9	64.7 $\pm$ 5.4	18.7 $\pm$ 0.9	29.6 $\pm$ 0.5	9.1 $\pm$ 0.6
INV-ODN	57.1 $\pm$ 3.5	26.3 $\pm$ 3.0	66.0 $\pm$ 1.0	18.1 $\pm$ 2.3	32.0 $\pm$ 2.4	8.7 $\pm$ 0.5
β_1 -AS-ODN	37.0 $\pm$ 1.0*	19.8 $\pm$ 1.8*	60.5 $\pm$ 3.4	10.0 $\pm$ 1.2*	30.7 $\pm$ 3.3	9.9 $\pm$ 0.5

β_1 - and β_2 -AR levels were measured 4 days after a single i.v. injection of saline, INV-ODN or β_1 -AS-ODN. Data represent mean $\pm$ SEM of each group (n=6-10). * $P<0.01$ versus saline control.

Effects on cardiac contractility and heart rate in response to β -stimulation

48 hrs after injection of 1mg/kg β_1 -AS-ODN, the cardiac inotropic and chronotropic responses to β -stimulation were determined in SHR *in vitro* and *in vivo*.

First, isolated hearts were perfused with incrementing doses of isoproterenol (ISO, nonselective β -agonist), which enhanced HR and contractility via activating β -AR. The dLVP-ISO dose-response curve, which reflected the positive inotropic effect of ISO, was significantly shifted down by β_1 -AS-ODN ($P<0.05$). HR was not significantly decreased, except at one point, i.e. 0.01 μ M ISO ($P<0.05$) (Fig. 5-2).

In vivo test was performed in conscious SHR monitored by radiotelemetry. β_1 -AS-ODN significantly ($P<0.02$) dampened the increase in $dP/dt_{\max}$ (index for cardiac contractility) in the face of dobutamine (β_1 -selective agonist) (Fig. 5-3A). The change in HR was not significantly reduced (Fig. 5-3B), which echoed the results in isolated perfused hearts. On the other hand, the response of BP to dobutamine was biphasic (Fig. 5-3C). Dobutamine elevated systolic BP by 5-8mmHg at low infusion speed and reduced systolic BP at higher speed. This is probably due to the partial β_2 -agonistic activity of dobutamine at high doses and the consequent vasodilatory effect on BP. Fig. 5-3 also compares the results with β_1 -AS-ODN and atenolol (β_1 -selective antagonist). Relative to β_1 -AS-ODN, 1mg/kg atenolol produced a more profound decline in $\Delta dP/dt_{\max}$ and ΔHR during 5-hr period of time after injection. Moreover, it reduced basal $dP/dt_{\max}$ (2450 ± 295 mmHg/sec versus control 2937 ± 277 mmHg/sec) and caused bradycardia ($295\pm$

12 bpm versus control 365 ± 8 bpm) ($P < 0.05$), while β_1 -AS did not change basal contractility (2922 ± 249 mmHg/sec) or HR (365 ± 12 bpm). However the effects of atenolol on $\Delta P/dt_{\max}$ and ΔHR were transient. Within 24hrs after atenolol administration, the inotropic and chronotropic effects of dobutamine had returned to control level.

5.2.2 Suppression of Peripheral Renin-Angiotensin System

Effects on renal β -AR levels

Scatchard analysis of β -AR binding in renal cortex (Fig. 5-4) indicated that β_1 -AR was the major subtype in the control rats, composing 70% of total β -AR. After β_1 -AS-ODN injection, the $B_{\max}$ of β_1 -AR was significantly diminished by 35% on day 4 ($P < 0.05$), 29% on day 10 ($P < 0.05$), 23% on day 18 and completely resumed on day 40. β_1 -AR reduction in kidney coincided with that in heart, which were accompanied by the significant drop in BP ($P < 0.01$). In contrast, the β_2 -AR level was not affected, nor the affinity of either subtype (data not shown). INV-ODN had no effect on either subtype.

Kidney slices from the same rats were subject to quantitative autoradiography in order to display the structural distribution of β -AR (Fig. 5-5). β_1 -subtype composed ~60% of β -AR levels, which was predominantly localized in renal cortex and outer band of medulla. β_2 -subtype was more diffusely distributed in the kidney at a lower level. This result was consistent with previous reports(205). Four days after β_1 -AS-ODN treatment, the overall density of β_1 -subtype in kidney was significantly reduced from 23.5 ± 2.1 to 15.4 ± 3.3 fmol/mg

($P < 0.05$). The diminution in renal cortex was particularly conspicuous due to the higher basal level. As expected, the distribution and concentration of β_2 -subtype remained unchanged in accord with binding results. This further confirms the specificity of the inhibitory effect of β_1 -AS-ODN on β_1 -subtype.

Effects on the peripheral renin-angiotensin system

RT-PCR (Fig. 5-6) revealed that the renin mRNA level in renal cortex was transiently decreased to 62% of control 4 days after β_1 -AS-ODN injection. It was completely reversed by day 18 (Fig. 5-7). On the other hand, PRA and plasma Ang II levels showed different patterns of reduction, which were significantly decreased on day 10 and day 18 ($P < 0.01$), but not on day 4. Thus PRA and Ang II seemed to have a delayed action relative to the reduction in renin mRNA (Fig. 5-7).

5.2.3 Absence of CNS Effects

Quantitative autoradiography of 6-18 tissue slices in brain, heart and kidney was analyzed four days after intravenous β_1 -AS-ODN administration (Fig. 5-8). No changes in the distribution of β -AR in forebrain and brainstem regions were detected. This indicated the absence of antisense effect on the β -AR expression in CNS. However, β_1 -AS-ODN significantly ($P < 0.05$) reduced β_1 -AR density in cardiac ventricles (from 30.2 ± 2.1 to 20.6 ± 2.5 fmol/mg) and renal cortex (from 26.4 ± 3.1 to 17.4 ± 3.3 fmol/mg). This was consistent with the binding results. β_2 -AR was not affected in any tissues.

5.3 Discussion

The data presented above demonstrate that β_1 -AS-ODN effectively decreases β_1 -AR density in heart and kidney, which led to a significant reduction in cardiac contractility and plasma renin activity. These findings are consistent with the hypothesis that β_1 -AS, through the inhibition of β_1 -AR expression, is able to render heart, kidney and other tissues less sensitive to sympathetic activation, which is a major contributing factor in high BP.

A single injection of β_1 -AS-ODN effectively decreases the cardiac β_1 -AR density, which produces a rapid reduction in ventricular contractility and thereby in cardiac output in response to β -stimulation. Unlike β -blockers, β_1 -AS-ODN does not decrease HR. Hence bradycardia is unlikely to be involved in the antihypertensive mechanisms of β_1 -AS-ODN.

The inhibition of renin release is regarded as a primary mechanism second to a decrease in cardiac output for the antihypertensive effects of β -blockers. Many β -blockers can reduce PRA in patients and experimental animals (55,206). They are found to be more effective in patients with higher renin profiles (56,57). In my experiments, β_1 -AS-ODN effectively decreased PRA and Ang II chronically. But the decrease in PRA and Ang II did not occur until ~10 days after β_1 -AS-ODN injection (Fig. 5-7), in contrast to the rapid drop in cardiac output two days after injection (Fig. 5-2 & 5-3). Thus it appears that the effects of β_1 -AS-ODN on the kidney renin and the circulating renin-angiotensin system are more delayed than cardiac action. Therefore suppression of cardiac output may

account for the early phase of the antihypertensive effect of β_1 -AS-ODN while the inhibition of renin-angiotensin activity acts as the secondary mechanism underlying the sustained reduction of BP in SHR.

Plasma renin activity (PRA) is regulated in several steps: synthesis of renin in kidney, conversion of prorenin into active renin, and secretion of renin into the circulation. By decreasing β_1 -AR levels in kidney, β_1 -AS-ODN significantly reduced PRA and the subsequent plasma Ang II levels. This is unlikely through the inhibition of renin expression, because there is no chronic diminution of renin mRNA levels. Instead, β_1 -AS-ODN may exert its inhibitory impact on renin secretion or the conversion of inactive renin to active renin. This hypothesis is consistent with the observation that β -adrenergic stimulation of renin expression had a different time course than that of renin secretion (207,208). Further, β -blockers have been shown to reduce prorenin processing to active renin without changing total renin (prorenin + PRA) levels in plasma (55). A flaw in this study is the omission to measure the local renin concentration in kidney cortex, where the circulating renin comes from. This assay would have provided an insight in the differential effects of β_1 -AS-ODN on renin production and secretion.

In addition to β_1 -adrenergic activation, renin expression is also regulated by the negative feedback of ANG II. Therefore the reversal of renin mRNA levels on day 10 and day 14 after β_1 -AS-ODN treatment may be explained by the decreased plasma ANG II concentrations that exerted less inhibition on renin expression.

Antisense is known for the gene targeting specificity. Radioligand binding assay showed that β_1 -AS-ODN is specific in inhibiting β_1 -AR without any effect on β_2 -AR. Therefore β_1 -AS-ODN may be free of the side effects that are caused by non-specific β_2 -antagonism of β -blockers.

The blood-brain barrier (BBB) formed by capillary endothelia is only permeable to small lipophilic molecules with molecular weight <600 dalton [reviewed by Pardridge (209)]. Owing to their high hydrophilicity, ODNs undergo negligible transport through BBB and have very limited access to CNS (130). Coupling with liposomes increases the lipophilicity of DNA, but the large size of DNA/liposome complex (100-300nm) makes it unable to cross BBB. The cellular and organ distributions of DNA/liposome complexes with fluorescent labeling were previously studied in mice after intravenous injection and the results indicated that the complexes were primarily taken up by capillary endothelial cells in most of the peripheral organs including lung, heart, kidney and spleen, but absent in the brain (210). Although the brain retention of liposomes after peripheral administration was observed in some cases, it is due to entrapment within the brain microvasculature (211). In our study, autoradiography in brain revealed no detectable changes in the expression and distribution of β -AR after intravenous β_1 -AS-ODN injection. This provided further evidence that our antisense approach did not have CNS effects. It also suggested that the inhibition of central β -adrenergic system is unlikely to contribute to the antihypertensive action of β_1 -AS.

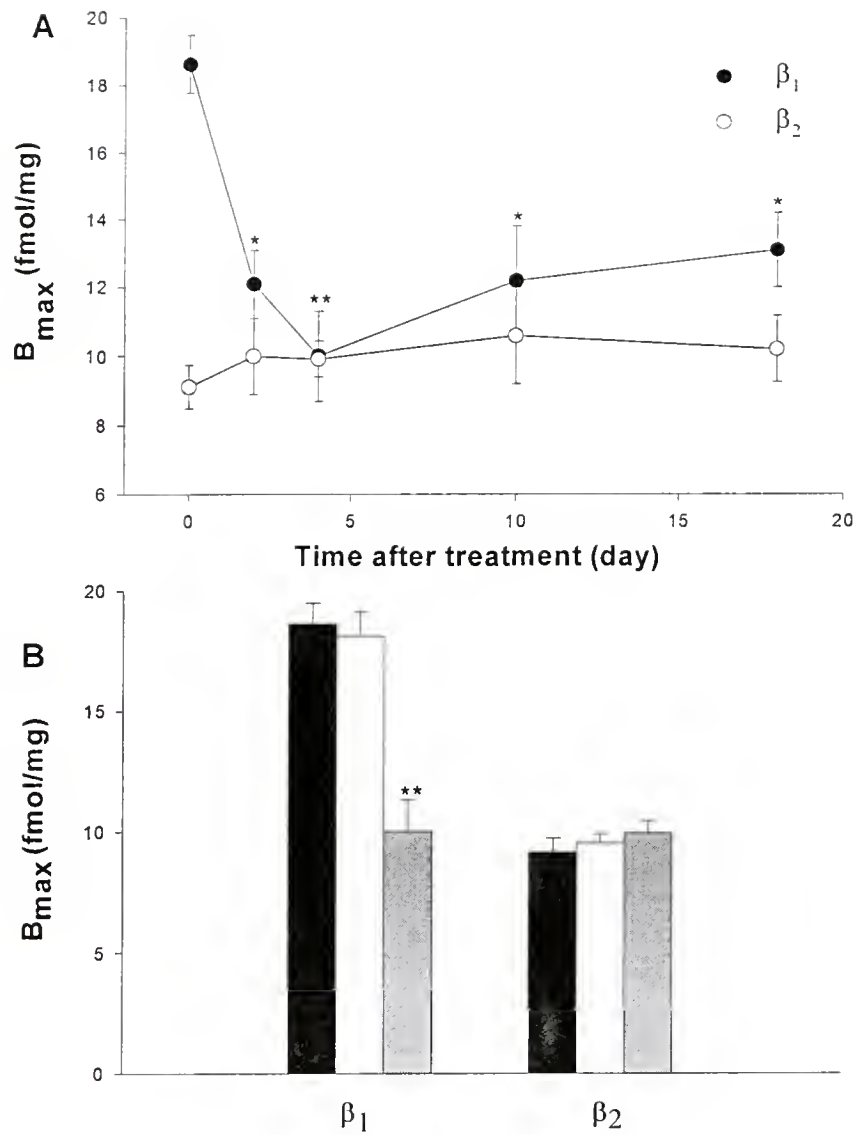


Figure 5-1. β_1 -AS-ODN decreased the density of β_1 -AR but not β_2 -AR in cardiac ventricles of SHR.

A). Time course of β_1 -AS-ODN effects on $B_{\max}$ of β_1 -AR (●) and β_2 -AR (○).

B). $B_{\max}$ of β -ARs 4 days after iv injection of saline (solid bar), 1mg/kg INV-ODN (open bar) or 1mg/kg β_1 -AS-ODN (shaded bar). Data represent mean $\pm$ SEM of each point (n=6). *P<0.01, **P<0.001 vs. saline or INV-ODN.

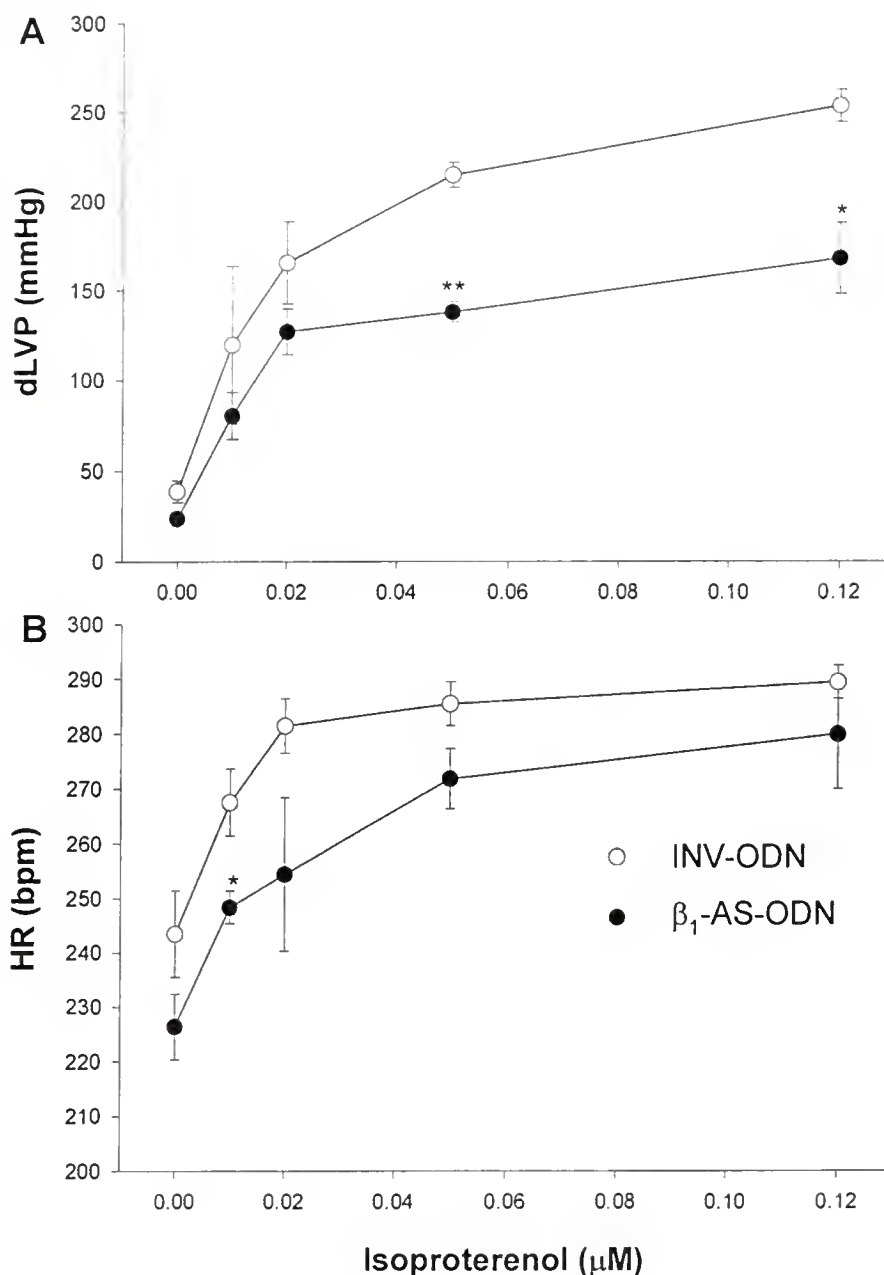


Figure 5-2. β_1 -AS-ODN reduced ISO-stimulated positive inotropic more than chronotropic response in isolated perfused SHR hearts.

48 hrs after iv injection of 1mg/kg INV-ODN (O) or β_1 -AS-ODN (●), hearts were perfused with Krebs buffer containing increasing concentrations of ISO. Shown is the effect of β_1 -AS-ODN on ISO-induced elevation in developed left ventricular pressure (A) and heart rate (B). The data represent mean $\pm$ SEM of each group (n=6-9). * $P < 0.05$, ** $P < 0.01$ vs. INV-ODN.

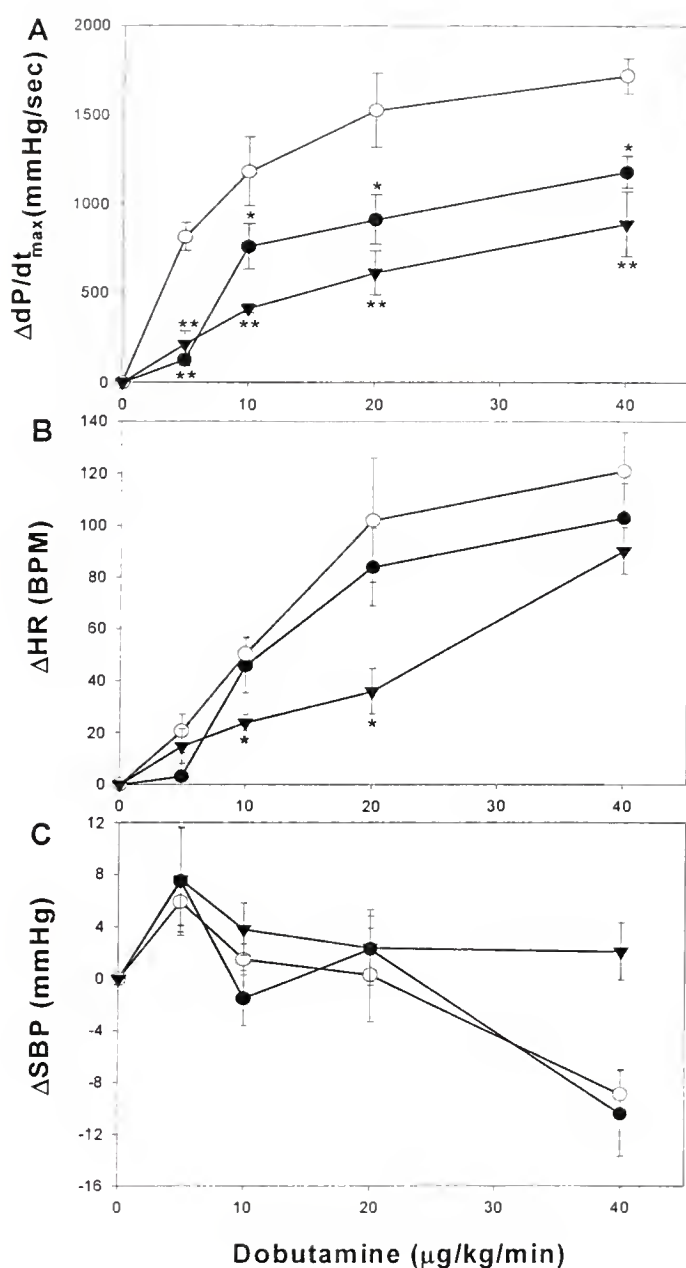


Figure 5-3. *In vivo* effects of β_1 -AS-ODN and atenolol on cardiovascular hemodynamics of SHR in response to β_1 -stimulation.

The same group of SHR ($n=4$) was tested for dobutamine-induced hemodynamic alteration at control levels (O), 48 hrs after β_1 -AS-ODN injection (●), and 1 hr after atenolol injection (▼). The rats were allowed to fully recover between treatments. During dobutamine infusion, cardiac $dP/dt_{\max}$ (A), heart rate (B) and systolic BP (C) of SHR were monitored by telemetry system. Data represent mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$ versus control.

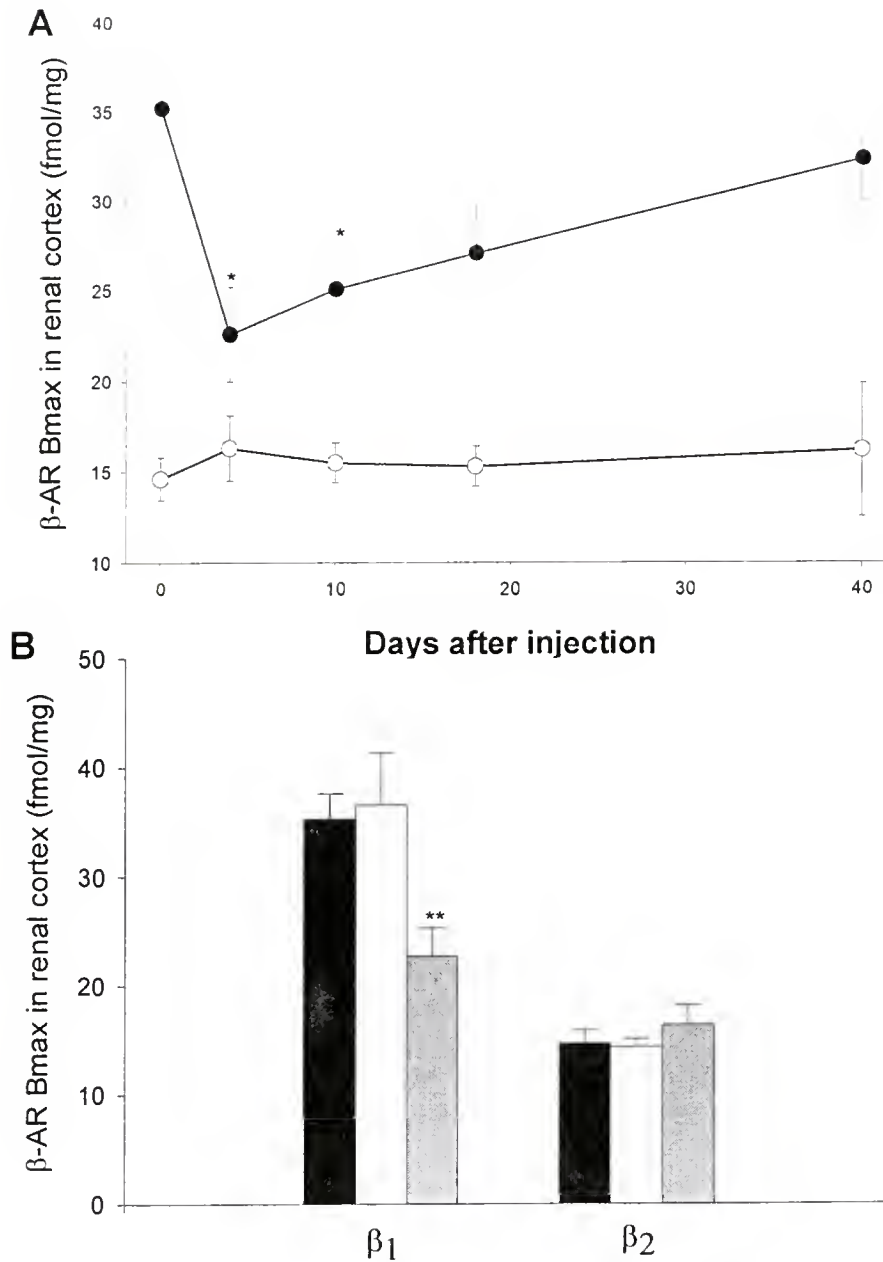


Figure 5-4. Effects of β_1 -AS-ODN on β -AR levels in renal cortex.

SHR were injected with 0.5 mg/kg β_1 -AS-ODN or INV-ODN with liposomes at charge ratio 2.0. A) Time course of the changes in $B_{\max}$ of β_1 -AR (●) and β_2 -AR (○). B) $B_{\max}$ of β -AR 4 days after iv injection of saline (solid bar), INV-ODN (open bar) or β_1 -AS-ODN (shaded bar). Data represent mean $\pm$ SEM of each point (n=6). * $P < 0.05$, ** $P < 0.01$ vs. saline or INV-ODN.

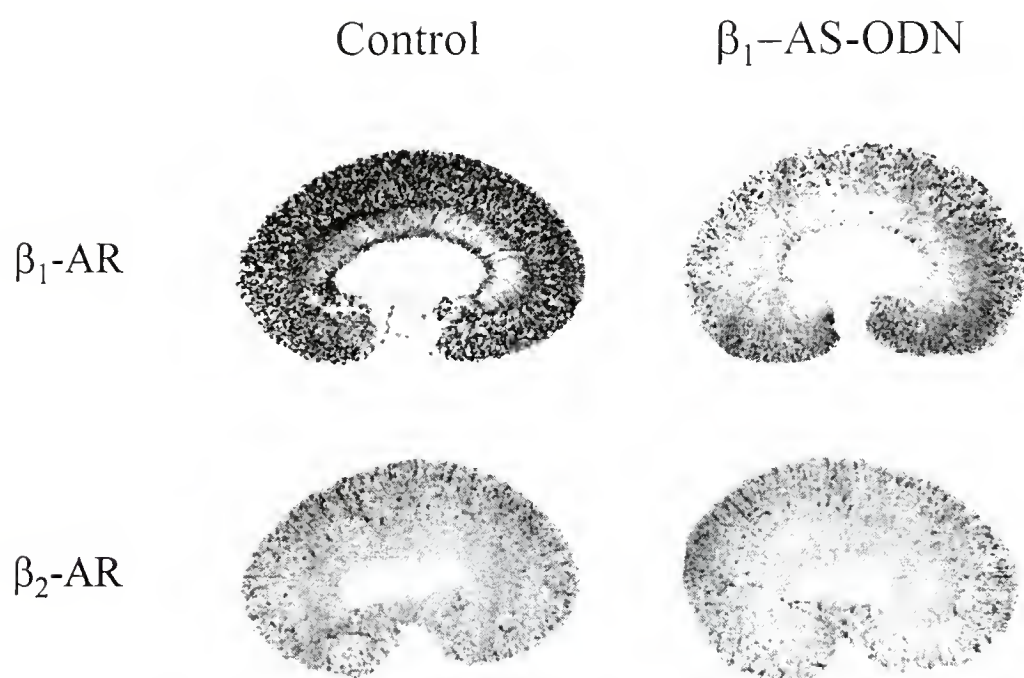


Figure 5-5. Representative autoradiography of β -AR levels in the kidney of control (n=6) or β_1 -AS-ODN (n=6) treated SHR 4 days after injection.

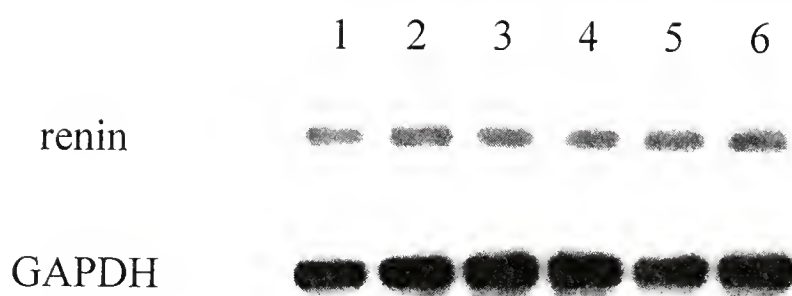


Figure 5-6. RT-PCR and DNA blot of renin and GAPDH mRNA in renal cortex of SHR.

Lane 1, saline control; 2, INV-ODN; 3, β_1 -AS-ODN day 4; 4, β_1 -AS-ODN day 10; 5, β_1 -AS-ODN day 18; 6, β_1 -AS-ODN day 40. Every point represents a pool of total RNA extracted from 4-5 rats.

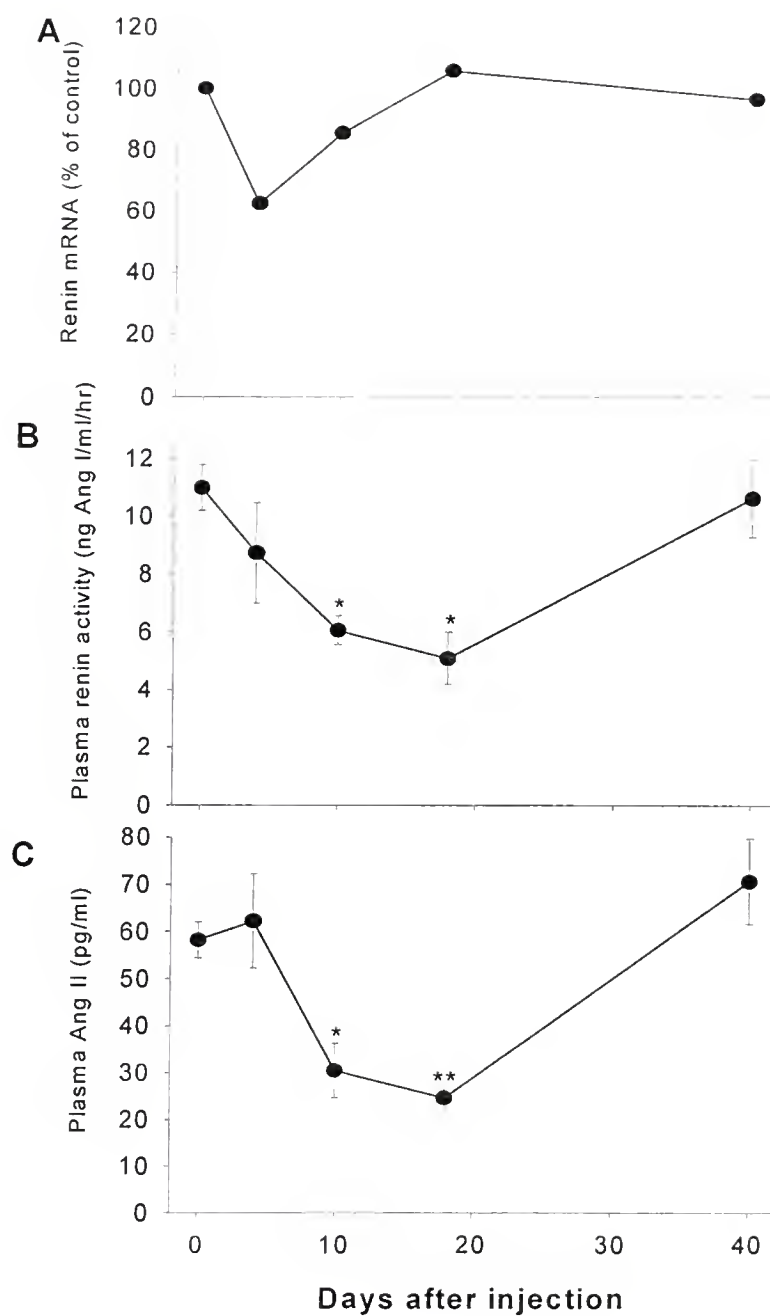


Figure 5-7. β_1 -AS-ODN at a single injection exerts a delayed suppression on renin-angiotensin system.

A, Effect on renin mRNA levels in renal cortex. *B*, Effect on plasma renin activity. *C*, Effect on plasma angiotensin II levels. Data represent mean $\pm$ SEM of each time point (n=6). * $P < 0.01$, ** $P < 0.001$ vs. baseline.

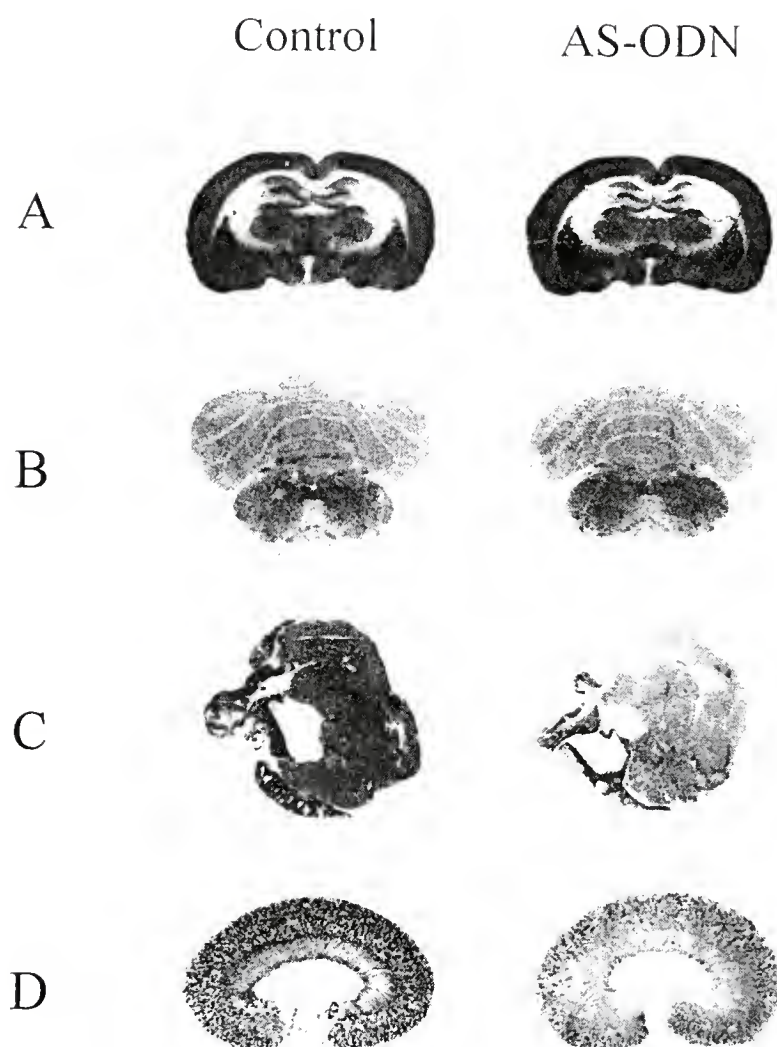


Figure 5-8. Autoradiographic illustration of β_1 -AR levels in different tissues of SHR.

A, forebrain; B, brainstem; C, heart; D, kidney. Autoradiography was performed 4 days after iv injection of INV-ODN (n=6) or β_1 -AS-ODN (n=6).

CHAPTER 6 REPEATED ADMINISTRATION OF β_1 -AS-ODN AND SAFETY PROFILE

6.1 Introduction

To be therapeutically useful, the β_1 -AS-ODN has to be effective with repeated administration and non-toxic. Currently there are at least ten clinical trials testing AS-ODNs in a variety of diseases [reviewed by Agrawal (137)]. These clinical data have convincingly demonstrated the sustained efficacy with repeated iv injection of AS-ODNs. Nucleic acids are poor epitopes. Human bodies usually do not develop immune response to nucleic acids except in rare autoimmune diseases. This may explain why AS-ODNs maintain effectiveness during chronic treatment.

Hypertension is associated with pathophysiological changes such as left ventricular hypertrophy (LVH) (212,213). Long-term antihypertensive therapies including angiotensin-converting enzyme inhibitors and β -blockers can prevent and reverse LVH (214). Therefore, if repeated treatment with β_1 -AS-ODN is effective in sustained reduction in high BP, it may as well inhibit the development of LVH.

Safety and pharmacokinetic profiles of several AS-ODNs have been reported. In these studies, phosphorothioate-ODN without any drug carriers were given through continuous iv infusion over 3-11 weeks and a wide range of doses

were tested for toxicity. Phosphorothioate-ODNs are well tolerated in humans at the doses < 2.5 mg/kg/day without any pathological or functional changes (215,216). The commonly seen side effects at higher doses are transient thrombocytopenia and fatigue (216,217). Likewise, studies in mice and monkeys revealed no adverse effects at doses $< \text{or} = 10$ mg/kg/day. Toxicity with large doses (>10 mg/kg/day) include splenomegaly, lymphoid hyperplasia and mononuclear cell infiltrates in mice, and complement activation and thrombocytopenia in monkeys (218,219). However, these adverse events are transient, reversible and only associated with high dosage (219-221). Lack of genetic toxicity has been confirmed by different researchers (164,219).

The polyanionic property of phosphorothioate-ODN due to the addition of sulfur atoms is responsible for some side effects such as thrombocytopenia (137). Due to the limited cellular uptake without appropriate vectors, AS-ODNs have to be given at high doses and frequency in these clinical studies. This in part accounts for the toxicity discussed above.

Liposomes are used as carriers for a wide variety of drugs. They are safe and non-immunogenic. They can also reduce the acute toxicity of many compounds such as amphotericin B and doxorubicin. Generally speaking, liposome formulations tend to protect tissues from the toxic effects of encapsulated compound and are usually better tolerated than the free drug (222). With improved uptake by liposomes, AS-ODN can be given at a lower dose to elicit the same therapeutic benefits. Cationic liposomes will also decrease the net negative charge of phosphorothioate-ODN and thus attenuate

its nonspecific interaction with serum proteins. Therefore cationic liposomes should be able to improve the delivery as well as reduce the toxicity of phosphorothioate-ODN.

This study is designed to test the effects of repeated injections of β_1 -AS-ODN on BP and cardiac hypertrophy in hypertensive rats. Toxicity and immune response will be assessed.

6.2 Results

6.2.1 Effects of Repeated β_1 -AS-ODN Treatment on BP

The efficacy of three repeated iv administrations of 1 mg/kg β_1 -AS-ODN (n=9) was tested in adult SHR with high BP. Animals treated with three repeated injections of 1mg/kg INV-ODN (n=7) or saline (n=7) served as controls. Systolic BP was measured every three days. A second injection of β_1 -AS-ODN was given before the BP returned to baseline after the previous treatment.

Three repeated injections of β_1 -AS-ODN at 16, 20 and 26 days maintained SBP of SHR at 20-30 mmHg lower than controls for more than 2 months (Fig. 6-1). There was no loss in the antihypertensive efficacy with repeated treatments. Instead, the 2nd and 3rd injections were more effective than the 1st one in the extended duration of BP reduction. Control animals treated with saline or INV-ODN had no change in BP with repeated injections. HR was not altered in any groups.

6.2.2 Effects of Repeated β_1 -AS-ODN Treatment on Cardiac Hypertrophy

At the end of 2-month treatment, SHR were sacrificed and the organs were weighed in a blind manner. The left ventricle of the heart was dissected free of fat and connective tissues, and weighed by a colleague who was not aware of the group assignment. Left ventricular hypertrophy (LVH) was indicated by the ratio of left ventricle weight to body weight (LV/BW). β_1 -AS-ODN treatment produced a small but significant ($P<0.05$) reduction in hypertension-associated LVH in SHR compared to saline control (Fig. 6-2).

6.2.3 Safety Profile

Liver Transaminases

Liver is a major organ for uptake and clearance of both liposomes and phosphorothioate-ODN when they are administered separately (222-224). Therefore large doses of liposome/ODN complex may overload the liver and cause liver toxicity. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are routinely used as a clinical index for liver function. Their levels increase substantially upon liver damage. Plasma concentrations of ALT and AST were measured after each injection, which revealed no significant difference between AS-ODN and control groups (Fig. 6-3).

Hematology

Phosphorothioate-ODN has increased negative charges due to sulfur atoms and may interact with positively charged proteins in the blood and cause hematological alterations. Reduced platelet counts were reported with high

doses of systemic injection of phosphorothioate-ODN (>5mg/kg/day for weeks) (225). At the end of 2-month treatment, hematological parameters were measured. There was no difference in total leukocyte count (WBC), platelet count, hematocrit and mean platelet volume (MPV) among three groups (Table 6-1).

Table 6-1. Clinical biochemical and hematological parameters after repeated administration of β_1 -AS-ODN.

	β_1-AS-ODN	INV-ODN	Saline
<u>Liver Enzymes</u>			
AST (unit/ml)	74.0 $\pm$ 3.7	83.0 $\pm$ 8.3	82.3 $\pm$ 3.8
ALT (unit/ml)	25.7 $\pm$ 1.1	30.5 $\pm$ 3.8	30.0 $\pm$ 3.2
<u>Hematology</u>			
hematocrit (%)	46.2 $\pm$ 0.63	46.0 $\pm$ 0.1	46.6 $\pm$ 0.33
WBC ($10^3/\text{mm}^3$)	5.48 $\pm$ 0.41	5.75 $\pm$ 1.15	5.86 $\pm$ 1.12
platelet ($10^3/\text{mm}^3$)	685 $\pm$ 12	706 $\pm$ 28	685 $\pm$ 22
MPV (fL)	6.93 $\pm$ 0.08	7.2 $\pm$ 0.20	7.0 $\pm$ 0.09
<u>C-Reactive Protein</u>	ND	ND	ND
<u>Antibody to ODN</u>	ND	ND	ND

n = 7-9 for each group, ND: non-detectable.

Immune Response

In order to investigate whether animals develop immune responses against repeated administration of β_1 -AS-ODN, gel immunodiffusion assay was employed to detect possible antigen-antibody reaction between liposome/ODN

complex and serum. If the body produces specific antibodies against a certain antigen, the increase of IgG levels will have different time courses after the initial and following inoculations. Second encounter with the antigen will mount a more rapid and greater elevation in IgG production than the first response. Considering this immune memory, serum samples for antibody detection were collected at 13, 7 and 7 days after the 1st, 2nd and 3rd injection, respectively.

No antibodies to ODN/liposome were detected in immunodiffusion analysis. Presence of C-reactive protein (CRP) in the blood is a clinical indication for immune reaction after infection. This parameter was found to be negative in all animals before and after repeated injections of β_1 -AS-ODN, INV-ODN or saline. It provided further evidence for the absence of immune responses.

Tissue Histopathology

High doses of phosphorothioate-ODN were associated with hyperplasia of mononuclear/phagocytic cells in the reticuloendothelial (RE) system (225). Heart, liver, spleen and kidney were subjected to pathological examination at the end of the study, which revealed no significant sign of immunopathology or organ damage (Fig. 6-4 to 6-7). Mononuclear cell infiltration was occasionally observed in small areas of the heart, but this occurred in all three groups. It is probably due to the higher background immune response in SHR, compared to WKY rats.

Organ Weights

Enlargement of spleen and kidney has been reported in rats receiving high doses of phosphorothioate-ODN (219,225). In this study, no significant change was found in the weight of spleen and kidney of all groups (Table 6-2).

Table 6-2. Organ weights after repeated administration of β_1 -AS-ODN.

	β_1 -AS-ODN	INV-ODN	Saline
<i>Tissue Weight</i>			
LV/BW (g/kg)	$3.10 \pm 0.07^*$	3.26 ± 0.06	3.33 ± 0.04
spleen (g)	0.64 ± 0.03	0.65 ± 0.01	0.62 ± 0.1
kidney (g)	1.32 ± 0.03	1.34 ± 0.08	1.34 ± 0.06

n = 7-9 for each group

* P < 0.05 vs. saline control.

6.3 Discussion

Maintained Efficacy and Lack of Immune Response after Repeated Administration of β_1 -AS-ODN by Cationic Liposomes

Any therapy that induces immune response cannot be truly useful in clinic, because immune response not only increases the danger of acute treatment, but also renders repeated administration ineffective. Gene therapy is no exception. Tremendous efforts have been made to avoid immune reaction in gene therapy. These efforts have mainly focused on the choice and development of appropriate vectors. Among viral vectors, adenovirus and HSV are known for acute toxicity and immune response, albeit new generations of vectors have minimized viral genomes and are better tolerated. Retrovirus has high transduction efficiency, but is usually limited in dividing cells. AAV is considered a safe vector, because wild type AAV has not been associated with any pathology in humans. It has a broad infectivity and can produce long-term expression of therapeutic genes due to the ability to integrate into the host

genome. However it has its own limitations, i.e. difficulty in production and relatively low expression [reviewed in (152-154)].

Liposomes, as a nonviral vector, are safe and non-immunogenic. In this study, liposomal delivery of β_1 -AS-ODN maintained its antihypertensive effectiveness upon repeated administration. No immune response or significant toxicity was observed. These results suggest that the liposome formulation is technically feasible to achieve sufficient gene delivery and produce prolonged therapeutic benefits, which are considerably longer lasting than traditional medicines.

Inhibition of LVH by Repeated Treatment with β_1 -AS-ODN

LVH, the disproportionate overgrowth of muscle in the left ventricle, has historically been considered the natural evolution of hypertension. It is estimated that the structural remodeling of the heart, including LVH, develops in about 70% of persons with sustained hypertension (212,213). Hypertrophied cardiac muscle not only increases myocardial mass and workload, but also contains excess collagen and increased fibrosis. LVH is associated with higher cardiac risks and viewed as a strong predictor for heart failure and myocardial infarction (226-229). In light of the importance of LVH in cardiovascular physiology and patient prognosis, much attention has been paid to the use of therapies to reverse the process. Among the antihypertensive agents, angiotensin-converting enzyme inhibitors are found to be the most potent for reversal of LVH, followed in the order by calcium channel antagonists and β -blockers (214,230,231). β_1 -selective blockade is highly effective in reversing LVH during chronic treatment (232).

My results indicate that repeated injection of β_1 -AS-ODN is not only able to reduce high BP, but also attenuate the cardiac remodeling associated with hypertension. 2-month treatment results in a small but significant reduction in LVH of SHR. Increasing the treatment period may exert more marked effects, as greater LVH regression has been observed after longer treatment with β -blockers (232). Since LVH is a risk factor for heart failure and myocardial infarction, β_1 -AS-ODN may as well protect the heart from these cardiac events by inhibiting LVH. However, whether the beneficial effects of β_1 -AS-ODN on LVH is due to direct inhibition of myocyte growth or secondary to the BP reduction is not known.

Limitations of the Current Study

Generally, the clinical assessment of toxicity requires more extensive measurements of pathological and biochemical parameters than what were determined in this study. Therefore further experiments should be performed to provide a complete safety profile of β_1 -AS-ODN including blood sugar, lipid profile, and liver and kidney functions. In addition, the toxicity data presented here were obtained at least 7 days after each injection. Although there was no adverse effect beyond this time point, it is still possible that ODN/liposome treatment may cause some acute toxicity.

Conclusion

The results of this study demonstrate that β_1 -AS-ODN can be given repeatedly to achieve a sustained reduction of hypertension and reduces cardiac

hypertrophy without causing toxic effects or immune stimulation. These data, together with early results, show that β_1 -AS-ODN is viable as a potentially new prolonged antihypertensive agent with benefits on cardiac remodeling.

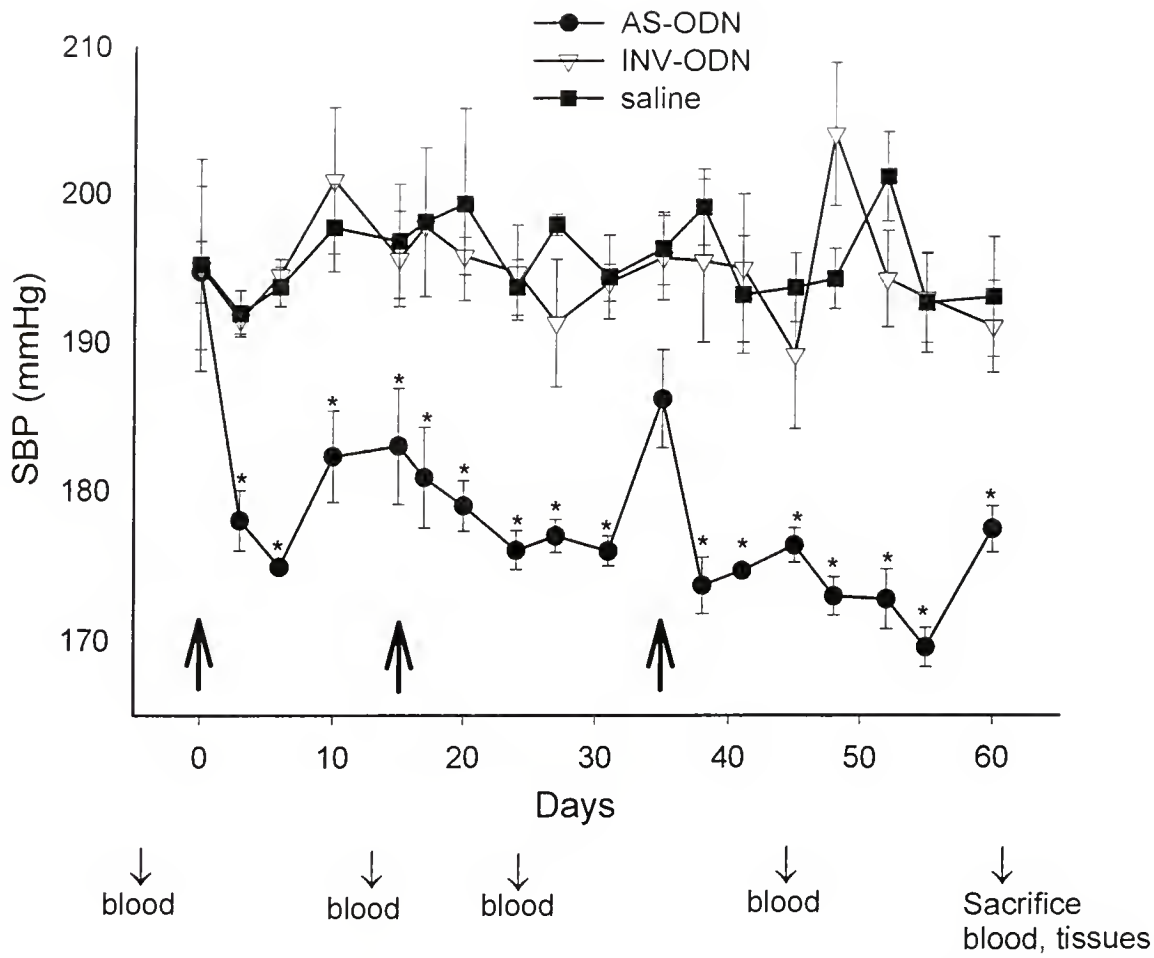


Figure 6-1. Sustained antihypertensive effects of repeated administrations of β_1 -AS-ODN in SHR.

Three repeated iv injections of 1 mg/kg β_1 -AS-ODN (n=9) delivered by liposomes were given to adult SHR as indicated by upward arrows. Repeated injection of saline (n=7) and 1 mg/kg INV-ODN (n=7) served as controls. Systolic BP was measured by tail cuff method. Blood samples were collected at different time points as indicated by downward arrows for safety profile analysis. Data represent mean $\pm$ SEM. * P<0.05 vs. saline or INV-ODN.

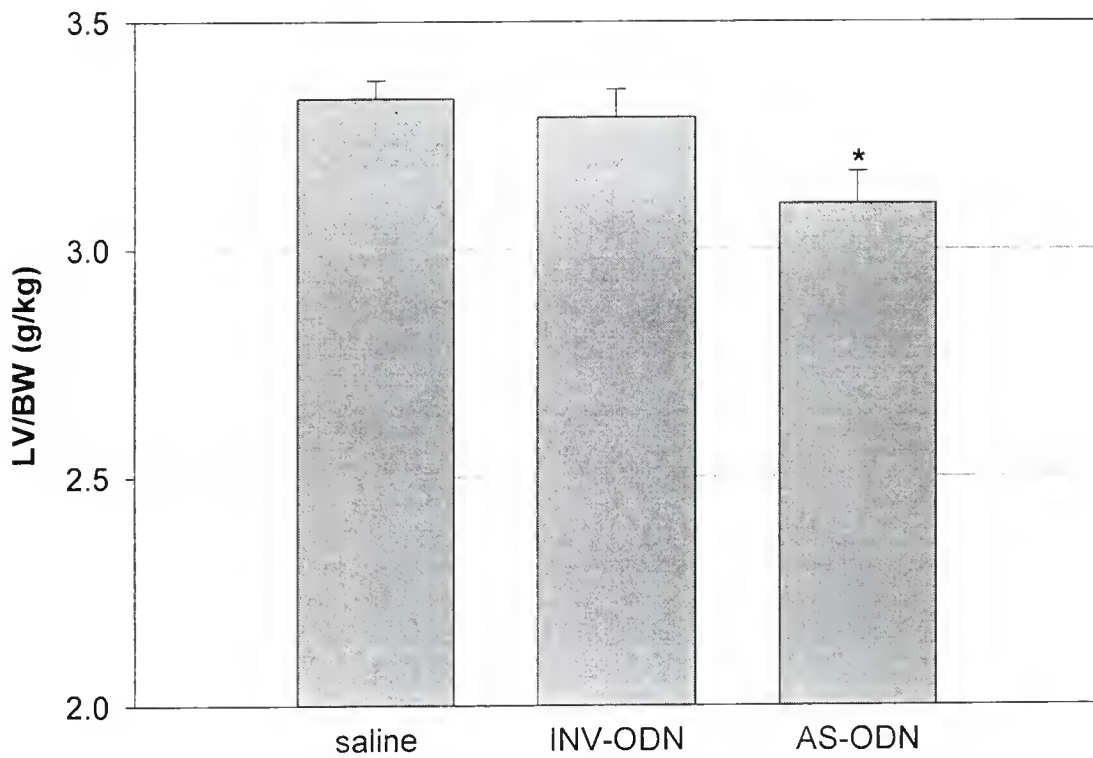


Figure 6-2. Repeated administration of β_1 -AS-ODN reduced left ventricular hypertrophy in SHR.

SHR were treated with repeated injections of β_1 -AS-ODN (n=9), saline (n=7) or INV-ODN (n=7) for 2 months. * $P < 0.05$ vs. saline.

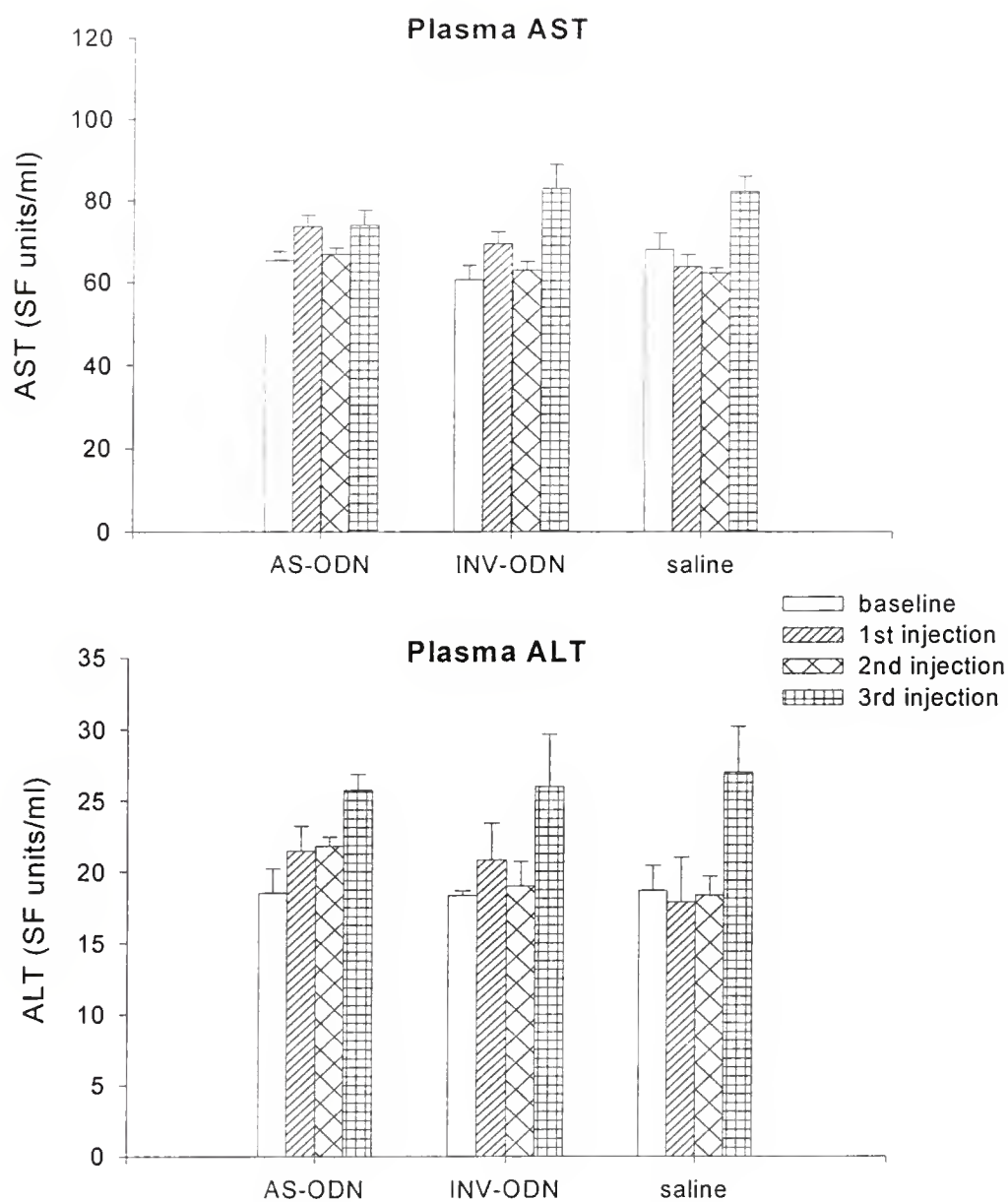


Figure 6-3. Plasma levels of liver transaminases ALT and AST after repeated injections of β_1 -AS-ODN.

Saline and INV-ODN serve as controls. Data represent mean $\pm$ SEM of each group (n=7-9).

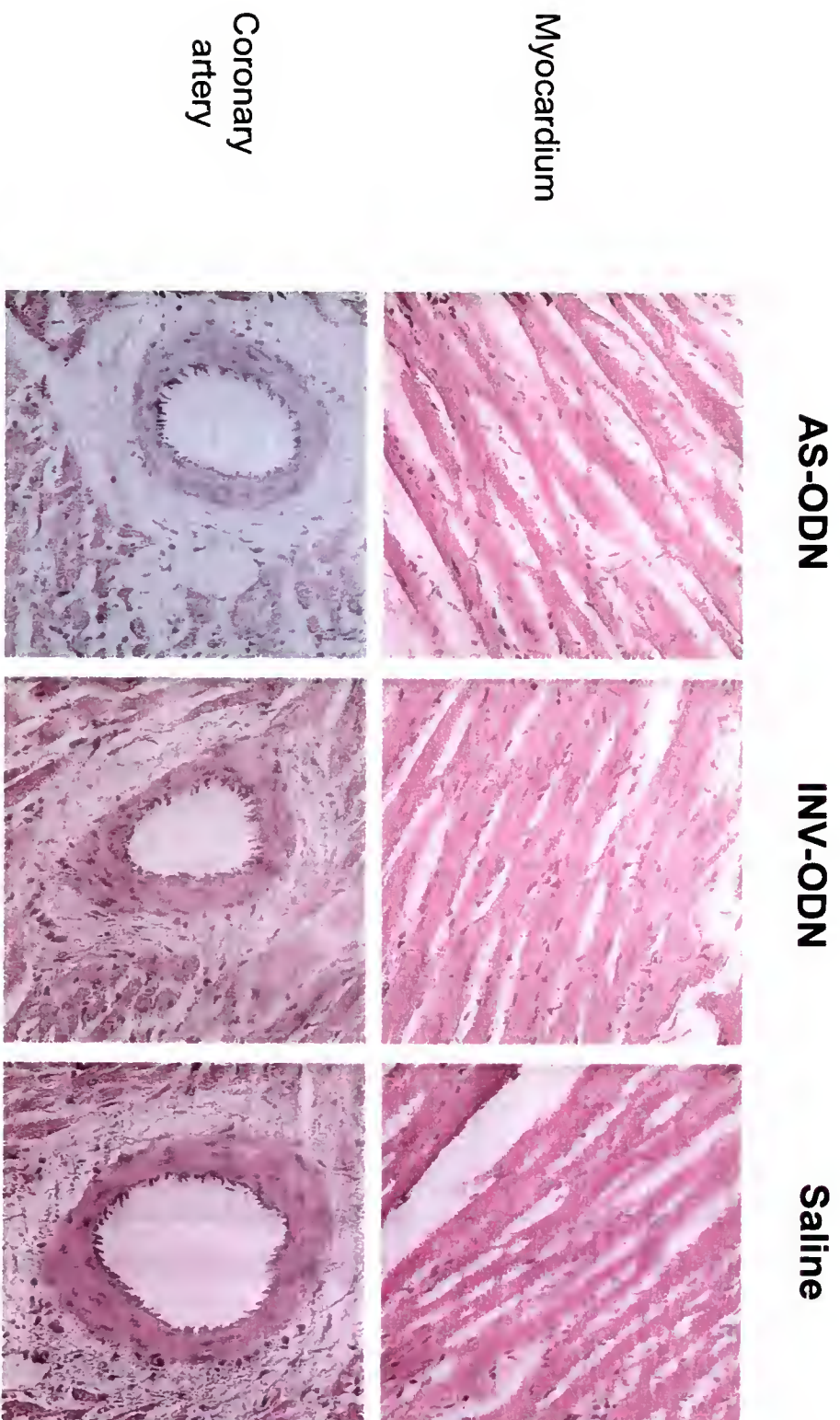


Figure 6-4. Histology of the heart from SHR after 2 months of treatment with β_1 -AS-ODN, INV-ODN or saline.

Hematoxylin & eosin staining. Magnification: $\times 400$.

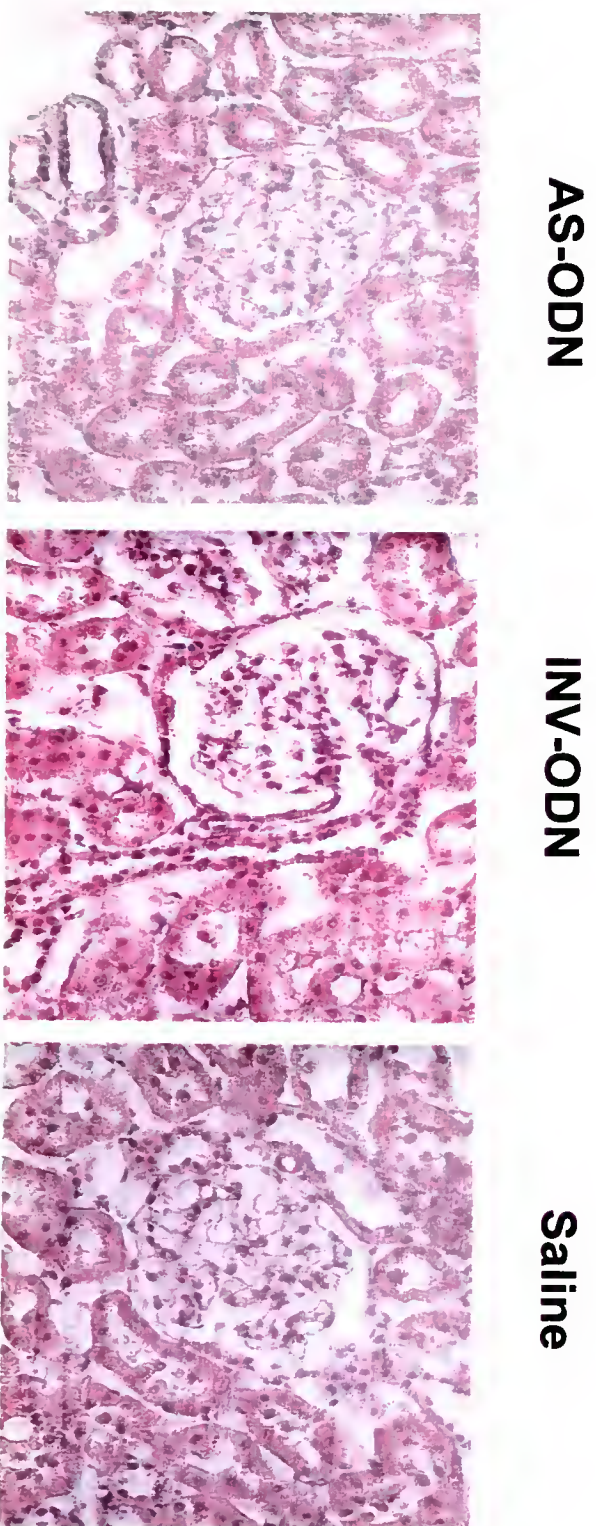


Figure 6-5. Histology of the kidney from SHR after 2 months of treatment with β_1 -AS-ODN, INV-ODN or saline.
Hematoxylin & eosin staining. Magnification: $\times 400$.

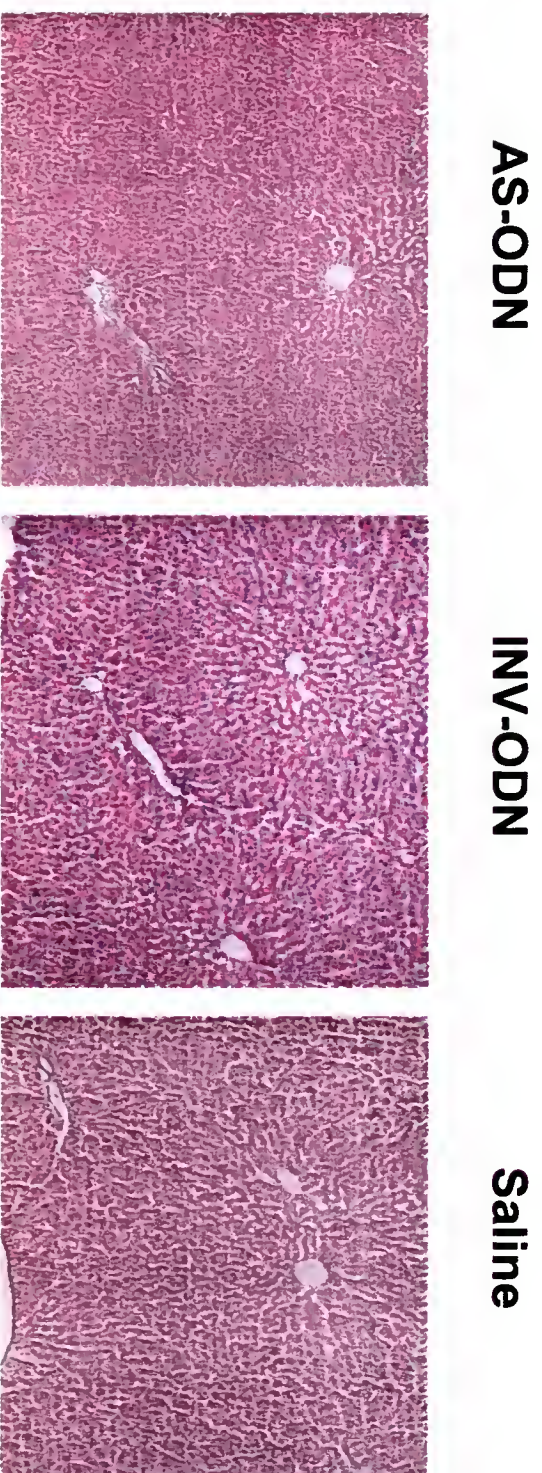


Figure 6-6. Histology of the liver from SHR after 2 months of treatment with β_1 -AS-ODN, INV-ODN or saline. Hematoxylin & eosin staining. Magnification: $\times 200$.



Figure 6-7. Histology of the spleen from SHR after 2 month treatment with β_1 -AS-ODN, INV-ODN or saline. Hematoxylin & eosin staining. Magnification: $\times 200$.

CHAPTER 7

PROTECTION BY β_1 -AS-ODN AGAINST MYOCARDIAL DYSFUNCTION INDUCED BY GLOBAL ISCHEMIA-REPERFUSION

7.1 Introduction

Acute myocardial ischemia causes a significant increase in plasma catecholamine levels, which leads to exacerbation of the myocardial injury and dysfunction. Acute myocardial ischemia is also characterized by increased sensitivity of β -ARs to catecholamines (233). β_1 -AR, being the predominant subtype in the myocardium, forms an interface between sympathetic nervous system and heart function. Several experimental studies show that the density and mRNA of β_1 -AR are augmented in the myocardium after acute ischemia (86,234). β -blockers, especially β_1 -selective blockers, can protect myocardium against ischemic injury and dysfunction (235), decrease infarct size (236,237) and reduce the incidence of sudden cardiac death after myocardial infarction (85).

The previous results have shown that β_1 -AS-ODN can inhibit β_1 -AR expression specifically and reduce BP in SHR for a prolonged period with a single iv injection. This study is designed to test the hypothesis that the use of β_1 -AS-ODN may protect myocardium against dysfunction in ischemia-reperfusion in the isolated rat heart, an *in vitro* model of myocardial infarction.

7.2 Results

7.2.1 Cardiac Dysfunction during Ischemia-Reperfusion

Four parameters were measured as the major indices for cardiac functions, including coronary perfusion pressure (CPP), left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP) and developed left ventricular pressure ($dLVP = LVSP - LVEDP$). Myocardial ischemia results in cardiac dysfunction, which is indicated by increased CPP and LVEDP and decreased LVSP and dLVP. An increase in CPP may result from an impaired coronary endothelium-dependent relaxation. The increased LVEDP and decreased LVSP represent insufficient contraction and relaxation of the myocardium. The net result of these changes is a diminished dLVP whereby the heart fails to pump enough blood to meet the elevated oxygen demand.

Five groups of SD rats were studied. Sham controls (n=6) were injected with saline and perfused continuously for 80 min without ischemia/reperfusion (I/R). In other groups, hearts were perfused for 20 min and subjected to global ischemia (30 min) followed by reperfusion (30 min). Saline + I/R (n=6) or atenolol + I/R (n=7) groups were injected iv with saline or 2mg/kg atenolol 6 hr before the hearts were excised. AS-ODN + I/R (n=7) or INV-ODN + I/R (n=7) were injected iv with 1 mg/kg β_1 -AS-ODN or INV-ODN 4 days before the I/R experiment.

The basal levels of CPP, LVEDP and dLVP were similar in all groups of rat hearts. In the sham controls, there were minimal (~5%) changes in the indices of cardiac function. In saline group, 30 min ischemia followed by 30 min

reperfusion resulted in marked cardiac dysfunction, indicated by a significant increase in CPP and LVEDP, and a decrease in dLVP ($P < 0.01$ vs. pre-ischemia values.)

Treatment with β_1 -AS-ODN markedly attenuated the ischemia-reperfusion induced ventricular abnormality, manifested by the preservation of dLVP and minimization of increase in CPP and LVEDP ($P < 0.05$ vs. saline group). Overall, AS-ODN treatment appeared to be equivalent to or even better than atenolol in these effects. AS-ODN restored dLVP to a greater extent than atenolol. INV-ODN showed no effect on ischemia-induced dysfunction and cannot be differentiated from saline group. Data on cardiac function parameters from these experiments are summarized in Fig. 7-1.

7.2.2 Malondialdehyde Levels in Myocardium

Malondialdehyde (MDA), a product of lipid peroxidation, has been used as an index of tissue injury after ischemia-reperfusion by several investigators (238;239). MDA levels in myocardium increased significantly after ischemia-reperfusion ($P < 0.05$ vs. sham control). Pretreatment of rats with AS-ODN and atenolol significantly attenuated this increase ($P < 0.05$ vs. saline treatment). As expected, INV-ODN did not affect MDA levels (Fig. 7-2).

7.2.3 β_1 -AR Levels in Myocardium

β_1 -AR levels in the left ventricle were measured after ischemia-reperfusion by radioligand saturation binding assay (Table 7-1). The Bmax of β_1 -AR was slightly elevated during ischemia-reperfusion, compared to sham controls. β_1 -AS-

ODN treatment significantly reduced the β_1 -AR density by nearly 50% ($P<0.05$), while atenolol or INV-ODN had no effect. β_2 -AR levels were not significantly changed in any group. The affinity of both β_1 - and β_2 -ARs remained unaltered.

Table 7-1. Bmax of β_1 - and β_2 -ARs in myocardium during ischemia/reperfusion.

	Sham	ischemia-reperfusion			
		Saline	INV-ODN	AS-ODN	Atenolol
β_1 -AR	21.5 $\pm$ 2.0	25.4 $\pm$ 4.4	26.5 $\pm$ 3.2	13.8 $\pm$ 0.9*	27.1 $\pm$ 4.0
β_2 -AR	11.2 $\pm$ 0.5	9.0 $\pm$ 0.5	10.2 $\pm$ 0.7	10.7 $\pm$ 0.7	9.7 $\pm$ 0.6

Data represent mean $\pm$ SEM of 7 rats per group. * $P<0.05$ vs. sham, saline, INV-ODN or atenolol groups.

7.3 Discussion

There is a generalized stimulation of sympathetic nervous system during myocardial ischemia, manifested by an increase in catecholamine levels in both plasma and myocardium (240-242), coupled with hypersensitized cardiac β -ARs (233). These changes are viewed as a compensatory response in an attempt to preserve cardiac function. However the consequence is detrimental, because the sympathetic activation increases the chance of tachyarrhythmia and workload of the heart which in fact exacerbates cardiac injury induced by ischemia. Consistent with this concept, pharmacological intervention of β -ARs can protect the heart and reduce mortality and morbidity during and after myocardial infarction (85), and prevent recurrent infarction (243). It is noteworthy that all the

β -blockers that have shown benefits in myocardial infarction patients can inhibit β_1 -AR, and no difference in the therapeutic efficacy has been found between β_1 -selective and non-selective blockers. This suggests that β_1 -AR is the major subtype mediating the deleterious effects of overactive sympathetic system on the heart. However, as discussed earlier, the use of β -blockers is limited by their intrinsic drawbacks including undesirable β_2 -antagonism and short action. A new strategy for specific β_1 -AR inhibition by antisense may overcome these problems and provide an effective and longer-lasting protection against myocardial ischemia.

Therefore, the present study was designed to examine the protective role of β_1 -AS-ODN against cardiac dysfunction after a brief period of ischemia-reperfusion. The effects of β_1 -AS-ODN was compared with those of a selective β_1 -blocker atenolol in this process. The results indicate that 30 min ischemia followed by 30 min reperfusion resulted in significant cardiac dysfunction and lipid peroxidation in the saline-treated rat hearts. Pretreatment with both β_1 -AS-ODN and atenolol preserved cardiac performance and reduced lipid peroxidation following ischemia-reperfusion. The protective effects of β_1 -AS-ODN on cardiac function were at least equivalent to those of atenolol, and AS-ODN seemed to provide a better restoration of dLVP than atenolol.

β_1 -AR, the predominant β -AR subtype in the myocardium, is the major mediator of sympathetic activation of cardiac functions. Several experimental studies have shown an upregulation of β_1 -AR, but not β_2 -AR levels, during acute ischemia (86,234). Ihl-Vahl *et al.* (86) convincingly demonstrated a subtype-

specific and time-dependent increase in mRNA levels of β_1 -AR during acute myocardial ischemia. This is consistent with the clinical data that point to the major role of β_1 -AR in ischemia-induced cardiac dysfunction. In the current experiments, a slight elevation in β_1 -AR levels was observed after brief ischemia-reperfusion, which did not reach the statistical significance. Reperfusion may explain the discrepancy here, because acute ischemia unequivocally increased β_1 -AR expression but the following reperfusion usually restored β_1 -AR to normal levels in several studies (87,234).

Both β_1 -AS-ODN and atenolol suppress β_1 -AR functions and protect the heart from severe injury after ischemia/reperfusion. However, these two approaches are different in several aspects. First, β_1 -AS-ODN reduced the total number of functional β_1 -AR on the cell membrane. Atenolol competed with endogenous catecholamines and did not affect β_1 -AR expression. Second, AS-ODN was given 4 days prior to ischemia-reperfusion to allow for sufficient reduction in β_1 -AR expression. Atenolol was given 6 hrs before excising the heart because of its rapid onset of action and short half-life. Therefore, β_1 -AS-ODN may provide a prophylactic protection from myocardial infarction in addition to its antihypertensive benefit. However it cannot replace the intravenous injection of β -blockers as the acute postinfarction rescue because of its slow effects.

Isolated rat, rabbit or guinea pig heart model provides an inexpensive and reproducible method to evaluate cardiac function and myocardial metabolic alterations during ischemia-reperfusion. It has been used extensively as an *in vitro* model for acute myocardial ischemia (182,239). In an isolated and

denervated heart, cardiac functions can be assessed independent of nervous and hormonal influences. Apparently, this simplicity has both advantage and disadvantage. Therefore further *in vivo* experiments are warranted for confirming the protective effects of β_1 -AS-ODN in acute and chronic myocardial ischemia.

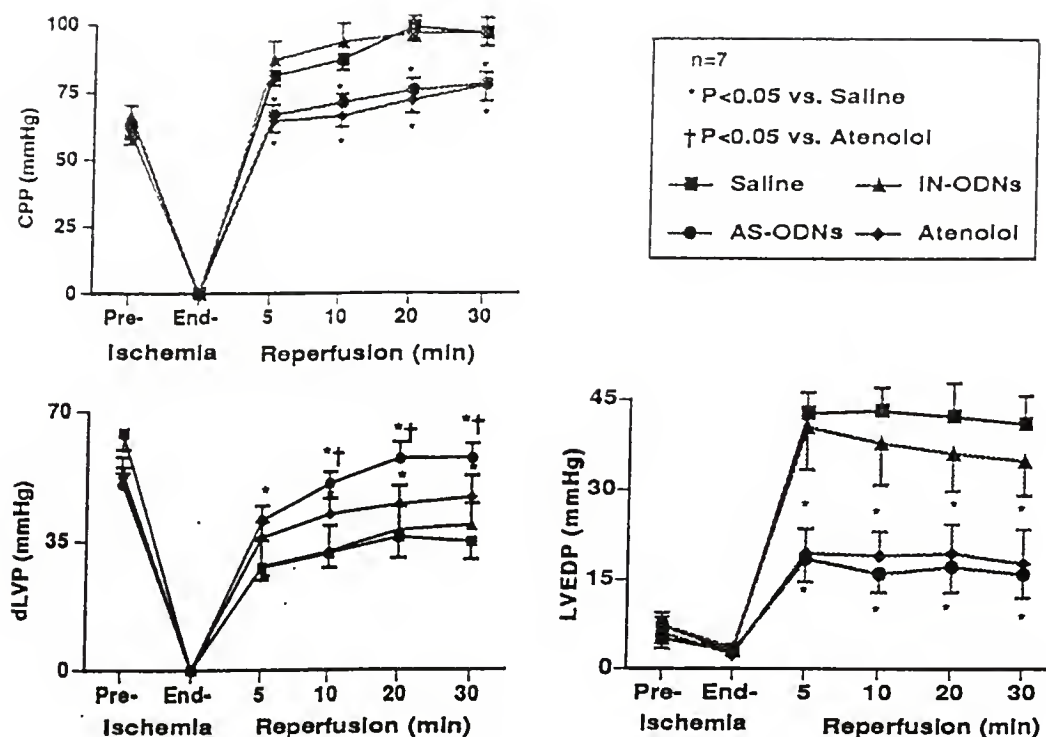


Figure 7-1. β_1 -AS-ODN and atenolol attenuated ischemia-reperfusion induced cardiac dysfunction.

Note the marked increase in LVEDP and CPP and decrease in dLVP in saline-treated rat hearts exposed to a brief ischemia-reperfusion. β_1 -AS-ODN, but not IN-ODN, exhibited beneficial effects on cardiac functions, indicated by a smaller increment in CPP and LVEDP and preservation dLVP. Atenolol showed a similar protective effect. Data represent mean $\pm$ SEM of $n=7$ for each group.

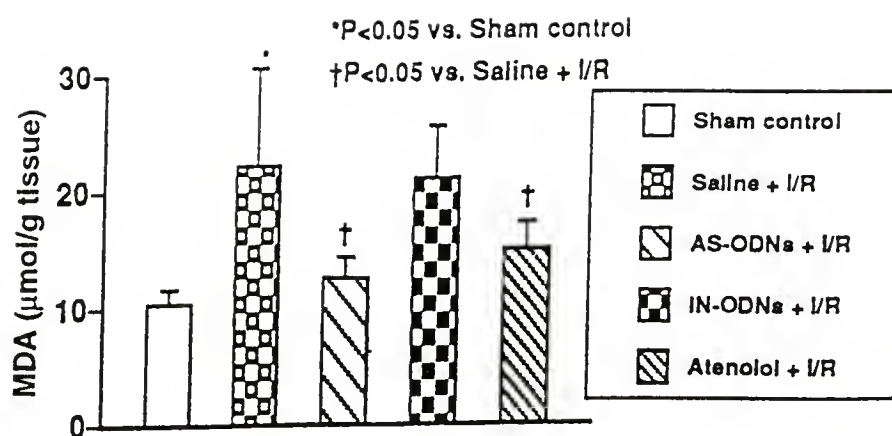


Figure 7-2. MDA levels in the myocardium.

MDA levels increased markedly after ischemia-reperfusion in the saline-treated rats. β_1 -AS-ODN, but not INV-ODN, attenuated the elevation in MDA. Data represent mean $\pm$ SEM of $n=7$ for each group.

CHAPTER 8 CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Conclusions

Cardiovascular diseases are the No. 1 killer in North America with a growing population of the elderly. Hypertension afflicts 50 million Americans and is a major risk factor for heart failure, myocardial infarction, stroke and renal dysfunction. β -blockers are the first line therapy for hypertension and effective in treating heart failure and myocardial infarction. However they have poor patient compliance due to daily regimen and side effects. Most of these side effects result from β_2 -adrenergic antagonism and CNS reaction.

The objective of this study was to develop a novel and specific β_1 -blocker using antisense gene therapy to treat hypertension and its complications with fewer adverse effects and prolonged benefits than traditional β -blockers.

A specific and effective antisense oligonucleotide targeted to β_1 -AR (β_1 -AS-ODN) was developed through *in vitro* and *in vivo* testing. It was delivered systemically by cationic liposomes DOTAP/DOPE. Under the optimal condition, a single injection of 0.5-1 mg/kg β_1 -AS-ODN produced a profound (25-35 mmHg) and prolonged (20-33 days) antihypertensive effect in a rat model of human essential hypertension. Repeated administration of β_1 -AS-ODN had maintained efficacy which not only reduced high BP persistently, but also attenuated

hypertension-induced pathophysiological changes such as left ventricular hypertrophy. β_1 -AS-ODN was also protective in a rat model of myocardial ischemia. It significantly reduced cardiac dysfunction and lipid peroxidation in the myocardium induced by ischemia/reperfusion. Chronic treatment with β_1 -AS-ODN was safe and well tolerated. No significant toxic effects or immune response were observed during 2-month treatment.

β_1 -AS-ODN is specific in inhibiting β_1 -AR expression without any effect on β_2 -AR, as evidenced by radioligand binding assay and quantitative autoradiography. It does not affect β -AR in the brain. Therefore β_1 -AS-ODN may avoid the common side effects of β -blockers with its β_1 -specificity and lack of CNS reaction.

β_1 -adrenergic activation increases cardiac output and stimulates renin release. The antihypertensive mechanisms of β_1 -AS-ODN appear to involve a rapid decrease in cardiac contractility and a delayed reduction in plasma renin activity and angiotensin II levels. It is likely that the suppression of cardiac output may account for the early phase of the hypotensive action of β_1 -AS-ODN while the inhibition of renin-angiotensin activity acts as the secondary mechanism underlying the sustained reduction in BP. Since β_1 -AS-ODN does not reduce heart rate or β -AR in the brain, bradycardia and central β -adrenergic inhibition are not involved.

In summary, β_1 -AS-ODN can be administered systemically with cationic liposomes to inhibit hypertension and cardiac hypertrophy and protect the heart from ischemic injury in rat models. Its antihypertensive effect is clearly longer

lasting than that of currently available β -blockers such as atenolol. Fewer side effects are expected with β_1 -AS-ODN because of its β_1 -inhibiting specificity and absence of CNS effects. Chronic treatment is safe without significant toxicity and immune response. These results demonstrate the potential therapeutic use of the antisense gene therapy using nonviral vectors for treating cardiovascular problems such as hypertension, cardiac hypertrophy and myocardial ischemia. The approach is conceptually solid and technically practical. It may be applied to other antihypertensive medicine that are currently used and offer an improvement in targeting specificity, fewer side effects and longer lasting therapeutic action.

8.2 Limitations of the Current Approach

Although β_1 -AS-ODN may offer significant improvements to β -blockers, its potential clinical application will be limited by the current route of administration, i.e. intravenous injection. Unlike synthetic compounds, DNA drugs including AS ODNs are under substantial degradation by digestive enzymes in the gastrointestinal tract and therefore cannot reach therapeutically effective concentrations in the circulation after the oral intake. Limited by the present technologies, an oral formula of DNA drugs has not been available. Considering the prevalent “needlephobic syndrome”, whether the intravenous injection of β_1 -AS-ODN will be accepted by patients remains a question.

8.3 Future Directions

This study has raised as many questions as it answered. For example, why β_1 -AS-ODN reduces cardiac contractility but not heart rate, whether the lack of decrease in heart rate is necessary for ameliorating cardiac dysfunction, and to what extent and by what mechanisms β_1 -AS-ODN can protect the heart from dysfunction and myocardial ischemia.

The absence of bradycardia after β_1 -AS-ODN treatment is the most surprising finding of this study, because it is well known that β_1 -AR mediates the positive inotropic and chronotropic responses of catecholamines in the heart. Most β -blockers can decrease heart rate. Hence this question seems to be a riveting one that deserves an answer. Several alternative hypotheses will be tested in the future experiments: 1) β_1 -AR may have a higher receptor reserve for controlling heart rate than contractility. 2) β_2 -AR may play a more important role in the regulation of heart rate than β_1 -AR. 3) Pacemaker cells in the heart may take up β_1 -AS-ODN less efficiently than cardiomyocytes.

Among many mechanisms proposed for the effectiveness of β -blockers in heart failure, one suggestion is that the decrease in heart rate may contribute to the protective effects on cardiac performance by reducing the workload and oxygen demand (73). However, since most β -blockers result in bradycardia, this mechanism has not been properly tested. Thus conflicting data surround this hypothesis. Some studies of β -blockers in human heart failure show that the greatest efficacy occurs in those patients with the highest pretreatment heart rates (67), whereas other reports did not find such a relationship (244). There are

β -blockers such as bucindolol that are effective in treating heart failure but have a low incidence of bradycardia (71). Now a β -blockade that decreases cardiac contractility and blood pressure without affecting heart rate can be readily achieved by β_1 -AS-ODN, which can be used to dissect out the role of heart rate in heart failure.

The results of this study indicate that β_1 -AS-ODN can inhibit left ventricular hypertrophy and preserve cardiac function after *in vitro* ischemia/reperfusion. This suggests the protective role of β_1 -AS-ODN in chronic heart failure and myocardial ischemia. Since these results were obtained in relatively short term experiments, more extensive studies are required to provide a convincing proof.

PATENT AND PUBLICATIONS

PATENT

MI Phillips, **YC Zhang**. U.S. Patent No. 6,087,343, entitled "Antisense oligonucleotides targeted to β 1-adrenoceptor and methods of use".

PUBLICATIONS

1. **Zhang, Y.C.**, J.D. Bui, L. Shen, and M.I. Phillips. Antisense inhibition of beta(1)-adrenergic receptor mRNA in a single dose produces a profound and prolonged reduction in high blood pressure in spontaneously hypertensive rats. *Circulation* 101: 682-688, 2000.
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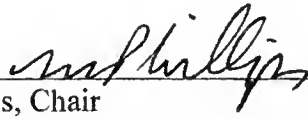
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BIOGRAPHICAL SKETCH

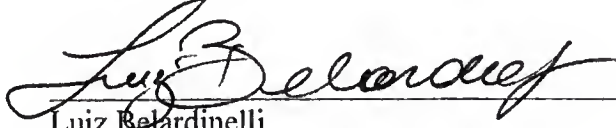
Yuan Clare Zhang was born on March 24, 1971 in Wuhan City of Hubei Province, People's Republic of China. Her father is Ming-wu Zhang, mother is Yi Feng. She went to elementary and middle schools in Wuhan. In 1988, after graduating from the high school, she entered Shanghai Jiaotong University majoring in Biochemical Engineering. She graduated with a Bachelor of Engineering degree in 1992. She then worked in the Shanghai Institute of Biochemistry, Chinese Academy of Sciences as a research assistant during 1992-1996. She came to the United States of America in 1996. She entered the Interdisciplinary Program of Medical Sciences in the College of Medicine, University of Florida in August 1996. After one year of core curriculum, she chose to perform her Ph.D. study under the guidance of Dr. M. Ian Phillips in the Department of Physiology. She has been studying gene therapy of cardiovascular diseases and has developed an antisense drug for treating hypertension. She has completed all the degree requirements and successfully defended her dissertation. She is expected to graduate and receive a Doctor of Philosophy degree in Physiology in August 2000.

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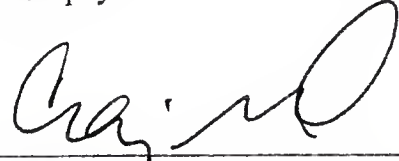
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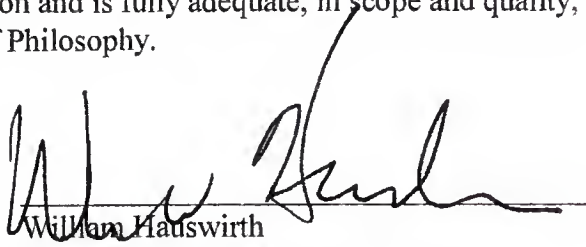
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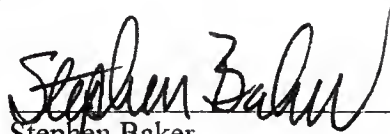
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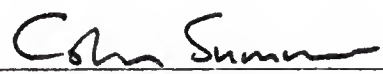
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Professor of Physiology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.



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